A thesis of the Degree of Doctor of Philosophy

Serological and molecular surveillance of *Orientia tsutsugamushi* in scrub typhus patients and coinfection of *Huaiyangshan banyangvirus*

쯔쯔가무시병의 혈청학적 분자생물학적 역학 조사 및 중증 열성 혈소판 감소증 바이러스 동시 감염 조사 연구

August 2020

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Seoul National University College of Medicine

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Serological and molecular surveillance of *Orientia tsutsugamushi* in scrub typhus patients and coinfection of *Huaiyangshan banyangvirus*

by

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A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Microbiology at Seoul National University, College of Medicine

August 2020

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ABSTRACT

1. Introduction: Scrub typhus is a mite-borne infectious disease caused by *Orientia tsutsugamushi*. *Huaiyangshan banyangvirus* (formerly Severe fever with thrombocytopenia syndrome virus (SFTSV)) is a tick-borne virus that can cause hemorrhagic fever. Severe fever with thrombocytopenia syndrome (SFTS) is emerging in Eastern Asia, including China, South Korea, and Japan, which are endemic region of scrub typhus. As the two infections overlap epidemiologically and clinically, knowledge on the local prevalence of the vector-borne infections is critical in target-oriented investigation and treatment.

2. Methods: To investigate the genotypic diversity of *O. tsutsugamushi* and potential coinfection with SFTSV in South Korea and Myanmar, 2,328 sera collected from patients with acute febrile illness in South Korea from 2000 to 2003 and 152 sera obtained from suspected patients with scrub typhus in Myanmar during 2018 were examined. I performed serological and molecular diagnosis of scrub typhus and SFTS. Phylogenetic analysis of both pathogens were also performed by sequencing of the amplified genes from *O. tsutsugamushi* and SFTSV.

3. Results: Among 2,328 samples from Korean patients, serological analysis detecting specific IgG against SFTSV NP antigen identified 37 positive samples (1.6%). Molecular detection of SFTSV *NP* gene and *O. tsutsugamushi tsa56* gene by PCR using the 37 specimens revealed 9 positive for SFTSV and 14 positive for *O. tsutsugamushi*, respectively. Sequencing of 14 *Orientia*-specific PCR products showed the presence of 3 genotypes, including Boryong (1/37, 2.7%), Karp (8/37, 21.6%) and Gilliam (4/37, 10.8%) genotypes. In addition, 4 specimens were positive for both SFTSV *NP* gene and *O. tsutsugamushi tsa56* gene, indicating coinfections. Among 152 Myanmar samples, 9 samples (5.9%) were positive for *O. tsutsugamushi tsa56* gene and sequencing of the PCR products revealed the presence of 5 genotypes, Karp A (4/9, 44,4%), Karp B (1/9, 11.1%), Kato B (2/9, 22.2%), Gilliam (1/9, 11.1%) and JG_C (1/9, 11.1%). 5 samples were positive for SFTSV *NP* gene-specific PCR (5/152, 3.28%). These 5 SFTSV *NP* gene positive samples were all

negative for *O. tsutsugamushi tsa56* gene, but 3 of them carried high titers (≥1:2,560) of IgG or IgM or both antibodies specific to *O. tsutsugamushi*, as measured by indirect immunofluorescence assay.

4. Conclusion: In this study, serological and molecular surveillance of sera collected from suspected scrub typhus patients in South Korea and Myanmar revealed serological prevalence and genotypic heterogeneity of *O. tsutsutsugamushi.* Moreover, potential coinfection of *O. tsutsugamushi* and SFTSV was identified by serological and molecular surveillance. These results suggest that SFTSV infections might have been spread more widely in Eastern Asia. Therefore, continuous surveillance of SFTS patients needs to be conducted in the local area. In addition, more reliable differential diagnosis techniques and prevention and control measures are required for better clinical practices and outcomes in the endemic regions of multiple tick-borne and mite-borne pathogens.

Keyword: *Orientia tsutsugamushi*, scrub typhus, Severe fever with thrombocytopenia syndrome virus, SFTSV, coinfection

Student number: 2014-25232

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LIST OF ABBREVIATIONS

BSA: Bovine serum albumin
cDNA: complementary DNA
DMEM: Dulbecco's Modified Eagle's Medium
E. coli : *Escherichia coli*
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal Bovine Serum
HFRS: Hemorrhagic fever and renal syndrome
HRP: Horseradish peroxidase
ICT: Immunochromatography
IFA: Immunofluorescence assay
IPTG: Isopropyl-D-thiogalactoside
LB: Luria-Bertani
Ni-NTA: Ni-nitrilotriacetic acid
OD: Optical density
PBS: Phosphate buffered saline
PBST: Phosphate buffered saline with Tween 20
PCR: Polymerase chain reaction
RT-PCR: Reverse transcription PCR
SD: Standard deviation
SFTSV: Severe fever with thrombocytopenia syndrome virus
TA: Thymine, Adenine
TMB: Tetramethylbenzidine

INTRODUCTION

Scrub typhus is a mite-borne febrile disease caused by an *O. tsutsugamushi* infection. The disease is endemic in Asian countries and the Western Pacific area, the 'Tsutsugamushi Triangle', with causing an estimated one million cases annually[1]. Recently, scrub typhus has also been reported outside of the endemic region, such as in South American and African countries. Scrub typhus is a neglected life-threatening disease that rivets the attention of missionaries, the military, and medical examination committees. Nevertheless, the global incidence of scrub typhus is poorly defined due to limited epidemiological data in many of the endemic countries[2]. Given that scrub typhus has been identified as one of the major causes of undifferentiated febrile illness, especially in the tropical region of Southern Asia[3][4][5], there is a raising need for epidemiological surveillance of the treatable infection to reduce disease morbidity and mortality.

O. tsutsugamushi was isolated in 1930, named for *Rickettsia Orientia*, and first reported in foreign soldiers in 1951 and 1986 in Korea. In the 1980s and 1990s, clinical and epidemiologic features were reported in nearly all university hospitals in each province in Korea. However, since 2000, the number of patients has increased but the number of clinical studies has decreased. The number of reported patients has been steadily increasing, the reporting system improving, and physicians' awareness increasing. The number of patients reported to the Korea Centers for Disease Control and Prevention increased from 1,000 to 2,000 in the early 2000s then to 4,698 in 2004. Subsequently, it increased sharply and reached 10,365 in 2013. The increase in outdoor activity and changes in the use of antibiotics may contribute to this change. Further, global warming had increased the density of mites, possibly leading to an increase in Tsutsugamushi disease.

The natural host for *O. tsutsugamushi* is the trombiculid mites in which the bacterial pathogen has maintained via transovarial or transstadial transmission in wild nature[8]. Since the pathogen is also associated with the salivary glands of chigger mites, it could be transmitted to the

vertebrate host while the infected mites feed on the tissue and fluid for their development. They maintain the infection throughout all of their life stages and as adults, and pass the infection on to their eggs in a process called transovarial transmission. Similarly, the infection passes from an egg to a larva or adult in a process called transstadial transmission. Similarly, the infection passes from the egg to the larva or adult in a process called transstadial transmission. In this way, chigger mite populations autonomously maintain their infectivity over long periods of time. Early workers thought that rodents were the natural reservoir of infection, but it is now believed that mites are both the vector and the reservoir. Clinical scrub typhus is not known to occur naturally in animals. This mite is fastidious in matters of temperature, humidity and food, and finds everything suitable in restricted areas. Scrub typhus is generally seen in people whose occupational or recreational activities bring them into contact with ecotypes favorable with vector chiggers. Small rodents and wild birds are the primary targets of chigger mites, and humans could be an accidental host[7]. The trigger bite is painless but an eschar often forms at the bite site. The disease symptoms are quite variable from mild to fatal illness in human infection. Early clinical manifestation of scrub typhus should be carefully evaluated with regional lymphadenopathy, followed by fever, headache, myalgia, and rashes. Due to a lack of specificity of its early clinical presentation compared to other acute febrile illnesses and an unavailability of rapid and effective diagnostic tests in local clinics and delayed treatment with suitable antibiotics, such as doxycycline and chloramphenicol, is common and often leads to severe complications including respiratory distress syndrome, acute renal failure, meningitis, gastrointestinal tract bleeding and multi-organ failures[6)7)8)9)]. The burden of disease is still quite large, accounting for up to 20% of febrile hospital admission in rural areas of Southeast Asia, and reaching a mortality rate of up to 10% depending on the area of endemicity[10)11)]. Moreover, the recurrent infection has often been often observed in the highly endemic regions[12)] potentially due to the antigenic diversity of *O. tsutsugamushi*[13)] and the failure to generate long-lived immunity after primary infection[14)].

A single sample with positive IgM antibodies has been associated with an acute/primary infection. Due to their long-term persistence in the blood,

the presence of IgG antibodies is useful in determining the prevalence of scrub typhus in a particular population. Seroconversion or a 4-fold rise in IgG titer using paired serum samples (acute and convalescent) better supports the diagnosis of scrub typhus[15]. Early serological assays, including the Weil-Felix and the complement fixation (CF) tests have the low sensitivity and specificity[16]. The indirect immunofluorescence assay test (IFA) was developed in 1963 in a direct IFA format using Karp and Gilliam strains for diagnosis of scrub typhus. The ability of the assay to identify the type of scrub typhus using Karp, Kato, and Gilliam strains with fluorescent antibodies in a direct IFA format was susequently demonstrated [17]. Karp, Kato, Gilliam,. Litchfield, the Japanese isolates (Irie, Hirano, Shimokoshi, Kawasaki, Kuroki, 432h-2, and Yamazaki), Thai isolates (TC586, TA678, TA686, TA716, TA763, and TH1817) and Boryong strains of *O. tsutsugamushi* have been used alone or in various combinations as antigens with in-house developed IFA assays and/or commercial kits[18]. Immunochromatographic rapid diagnosis tests (ICT) have been developed in the last decade following the need for rapid diagnosis tools for the diagnosis of scrub typhus. Additionally, the enzyme-linked immunosorbent assay (ELISA) using whole-cell antigens of Karp, Kato and Gilliam strains of *O. tsutsugamushi* has been shown to be useful for detecting antibodies against new species/strains of *O. tsutsugamushi* outside of the traditional Tsutsugamushi Triangle[19] [20][21][22].

O. tsutsugamushi infections were documented in Myanmar during the 1940s and scrub typhus was a major health problem during the Second World War II[23][24]. Since then, however, there have been no reports describing the prevalence and genetics of scrub typhus in Myanmar, although recent studies conducted on the Thai-Myanmar border identified scrub typhus as one of the primary infections causing acute febrile illness. They have also reported the recovery of two additional strains of *R. orientalis* from pools of mites collected from bandicoots *E. cockerelli* in Ramsay in the Dobadura area in New Guinea[25]. Umphang, the southernmost district of the Thai province of Tak, is situated at the Thai-Myanmar border and lies near the central point of the Tsutsugamushi Triangle. *Leptotrombidium* which transmitted *O. tsutsugamushi* to a host through the bite of an infected mite

of the genus can be found in many different types of vegetation (e.g. forests, rice paddies, or plantations). Farmers and people who engage in outdoor activities have a higher risk of contracting scrub typhus[26)27)28)]. This district is dominated by untouched nature, farmland, and mountainous terrain, and more than 90% of the registered population lives in a non-urban-environment with the majority of the population engaging in the farming industry. Hence, the demography and surrounding environment make this district at the Thai-Myanmar border an ideal setting for scrub typhus. This area harbors many unregistered people, such as refugees, migrant workers, and descendants from hill tribes, entailing a complicated humanitarian situation[29)]. Thailand's Sangkhlaburi District (Kanchanaburi Province) is a major gateway on the central part of the Thai-Myanmar border where newly arrived migrants from Myanmar become established as farm or factory laborers. There, the local Thai people, as well as Karen, Mon, and Burmese migrants are commonly bitten by arthropods when working in the fields or at home[30)]. These results underscore the need for research on the vector-borne infection in this area, including defining the genotype diversity of *O. tsutsugamushi*, which has been a serious obstacle for developing effective diagnostic methods and vaccines for scrub typhus.

Huaiyangshan banyangvirus (formerly Severe fever with thrombocytopenia syndrome virus (SFTSV)) was first confirmed in China in 2009, and retrospective serological studies showed that SFTSV-specific IgM and IgG were detected in the sera of patients in China in 1996 and retrospectively reported in South Korea in 2010, in Japan in 2013 and Vietnam in 2017[31)]. Some viruses in China and South Korea were transmitted, likely several times, from Japan across the East China Sea and/or the Sea of Japan, and vice versa. This fact was also supported by evidence that SFTSV infected patients were confirmed in Jeju Island, South Korea, and there were no discernible patients in the Honshu, Shikoku, and Kyusyu islands of Japan, which are separated by a sea. At present, there are other possibilities, such as the transport of humans and/or livestock accompanied by traveling and trade, because the cultural exchanges between China, South Korea, and Japan have been active for many centuries. In Vietnam, 2 SFTSV infected patients were confirmed in Hue in 2017 by amplifying the partial S segment of the viral RNA in stored serum from patients with

thrombocytopenia who had elevated levels of serum hepatic enzymes, including aspartate aminotransferase and alanine aminotransferase; and gastrointestinal symptoms, such as vomiting. *H. longicornis*, *A. testudinarium*, and *I. nipponensis* ticks are vectors of SFTSV and *A. testudinarium* has been found in Vietnam. Migratory birds are known to be long-distance carriers of virus-bearing ticks. Therefore, virus-bearing *A. testudinarium* ticks and migratory birds may play a role in the dispersal of SFTSV to Vietnam. Since then, in Myanmar there have been no reports describing the prevalence and genetics of SFTSV.

SFTSV belongs to the third group of the *Phenuiviridae* genus, which is a member of the *Bunyavirales* family. The genus *Phenuiviridae* currently includes over 70 antigenically distinct serotypes, 68 of the known serotypes are divided into two groups: *Phlebotomus fever* viruses which includes 55 members (transmitted by *Phlebotominae* sandflies) and the *Uukuniemi* viruses (transmitted by ticks) comprised of 13 members. Only eight species (*Alenquer*, *Candiru*, *Chagres*, *Naples*, *Punta Toro*, *Rift Valley fever*, *Sicilian*, and *Toscana* viruses) cause human diseases. SFTSV has been recognized as a novel member of the *Phlebovirus* genus because it is distantly related to both the existing *Uukuniemi* virus and *Phlebotomus* fever viruses[32].

The clinical course to the SFTS patients could be divided into three major stages starting with fever, followed by multi-organ dysfunction, and then the convalescent stage. The incubation period ranges between 1 to 2 weeks, leading to the fever stage which ranges from day 1 to day 7 post-onset of illness. This stage is characterized in both survivors and fatal cases by flu-like symptoms, thrombocytopenia and leukocytopenia, and a slight increase in serum biomarkers alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase, and creatine kinase isoenzyme. These symptoms may resolve after 1 week in survivors whose serum enzymes begin to decline toward normal levels and who then show signs of recovery 2 weeks after the onset of the disease. Later, some cases moved on to the multi-organ dysfunction stage, where the serum viral load decreased in nonfatal cases but remained high in fatal cases, while the platelet counts reverted to a normal value in nonfatal cases but continued to decline in fatal cases. In the third stage

(over 14 days), nonfatal cases were able to recover from the disease with most clinical parameters converting back to normal. In contrast, the fatal cases showed increasing serum enzymes accompanied by the main complication including multiple organ failure, coagulation, shock, acute respiratory distress syndrome, and so on[33)34)35)].

In the past decades, *Phenuiviridae* was mainly circulating in some parts of Africa and Europe, mainly the Arabian Peninsula, in the regions with the highest incidences of the *Phlebotomine* sandfly transmitted viruses. It seems most likely that *Phenuiviridae* involve arthropod vectors and mammalian hosts, including cats, mice, hedgehogs, weasels, possums, and yaks. SFTSV is transmitted from adult ticks to larva through ovarian transmission. Mammals, such as deer, and cocoons are infected with SFTSV through tick bites causing a transit viremia. Ticks acquire SFTSV by biting viremic mammals. SFTSV is maintained in nature through the close interaction between several species of ticks such as *H. longicornis* and *A. testudinarium,* and *I. Nipponensis* and mammals. Humans appear to be accidental hosts and play no essential role in the life cycle of SFTSV. Some studies indicated that ticks, especially *H. longicornis*, were the major potential insect vector to transmit the virus to humans. Although rodents are known as reservoir hosts of *Bunyaviruses*, there is a pronounced discrepancy on SFTSV in rodents. However, the disease can also be transmitted from person to person through contact with an infected patient' s blood or mucous. SFTSV is a new emerging tick-borne virus that can cause hemorrhagic fever with a high mortality of up to 40%. A recent molecular evolution analysis suggested that SFTSV originated 20 to 87 years ago in central China and then was transmitted to other endemic countries. Most SFTSV infections occur through bites from the tick *H. longicornis*, although SFTSV transmission can also occur through close contact with an infected patient.

Since the first report of SFTSV in humans, the number of human cases has rapidly increased each year in China, South Korea, and Japan[36)37)38)]. Although the average case fatality rate varies among regions and years [39)], the mean mortality rate of SFTSV cases has remained relatively high in Japan (27%), South Korea (23.3%), and China (5.3%-16.2%). No vaccines or effective drugs against SFTSV have yet been developed. The

severity and clinical outcome of patients infected by SFTSV were associated with viral replication and host immune response. Therefore, the control strategies for SFTSV are blocking transmission and protecting susceptible populations, including protecting against tick bites, avoiding direct contact with infectious blood or patients, using personal protective equipment when treating patients, and isolating patients and suspected clinical SFTSV patients[40)41)].

Early clinical diagnosis of SFTSV infection is mainly based on the patients' clinical manifestations and laboratory tests, such as fever, thrombocytopenia (and/or leukopenia), and elevated levels of liver enzymes, and epidemiological characteristics including the history of a tick bite, living and working environments and epidemic season[42)43)]. Confirmation of SFTSV infection requires one of the following conditions: isolation of SFTSV from the patients' samples, detection of SFTSV RNA in the patients' blood or serum, or detection of SFTSV-specific antibodies in the patients' serum. Virus isolation is often the most confident evidence for virus infection, and SFTSV could only cause CPE on DH82 cells, but not on Vero cells or Vero E6 cells in primary isolation, which may be why isolation of SFTSV has failed for such a long time in China[44)]. This method is mainly used in research institutions, however, the work is always very time and energy-consuming, so that the other two strategies are often applied in clinical confirmation assays. Commonly, the viral nucleic acids can be detected in the acute phase serum of patients, within 2 weeks post-onset of the disease. Several methods have been developed to amplify the viral RNA, including reverse transcription loop-mediated isothermal amplification (RT-LAMP). Recently, a method that incorporates RT-cross-priming amplification (RT-CPA) coupled to a vertical-flow visualization strip has been developed for the rapid detection of SFTSV, which is even more simple and near-instrument-free, and very practical for point-of-care testing[45)46)47)]. Several serological tests have been used to detect antibodies against SFTSV. Double antigen sandwich ELISA, in house Mac-ELISA and indirect ELISA assays were developed to detect SFTSV specific total, IgM, and IgG antibodies in serum samples, respectively[48)]. The indirect immune-fluorescence assay is also used to test the virus-specific antibodies from SFTSV infected cells or tissues,

which is often used for confirmation of virus infection and observation of infectious status[49].

Mixed infection with SFTSV and *O. tsutsugamushi* has been detected in South Korea where both pathogens are endemic. These results further emphasize the urgent need for an epidemiological study on the vector-borne diseases in the area of endemicity to improve our stability to accurately differentiate and properly treat these febrile infectious diseases with atypical signs and symptoms during the initial stage[50]. The clinical presentations of SFTSV and scrub typhus are similar during the early stage of infection: signs and symptoms typically develop within 1 and 2 weeks of infection and include fever, headache, malaise, and gastrointestinal symptoms, but SFTS exhibits a higher mortality rate than scrub typhus[51]. In South Korea, SFTSV and scrub typhus are endemic and are major public health concerns between May and November and between October and November, respectively. Mixed infections with SFTSV and *O. tsutsugamushi* in a patient have been reported.

SFTSV and *O. tsutsugamushi* are often neglected tropical diseases bearing similar seasonal patterns and rodents as a common reservoir. Their common epidemiology creates an opportunity for dual infections. Although SFTSV and *O. tsutsugamushi* do not share a specific vector, they are transmitted to humans through tick and mite bites mostly, respectively, during outdoor activities. The ecological differences between vectors may characterize their epidemiological features, including the region of infection and peak epidemic seasons. However, there are considerable overlaps of their epidemiological and clinical features, which makes their differential diagnosis difficult, particularly during the high epidemic season of scrub typhus. A high index of suspicion by primary physicians is needed in patients of endemic regions presenting with acute febrile illness, myalgia, or a rash which may be suggestive of coinfection with scrub typhus and SFTS.

In this study, I provide retrospective evidence of SFTSV infections or mixed infection with *O. tsutsugamushi* in patients' sera collected in South Korea from 2000 to 2003. These findings confirmed that SFTSV infections in South Korea occurred earlier than reported in South Korea and Japan and were more concurrent with those in China. Considering SFTSV

infection in patients with scrub typhus is also important for clinical diagnosis.

I investigated the serological prevalence and genotypic diversity of *O. tsutsugamushi* using blood samples from suspected scrub typhus patients in Myanmar. I also searched for possible infection of SFTSV which has been a new emerging threat to public health in Eastern Asia. My current serological and molecular survey on these vector-borne infections revealed that scrub typhus is highly prevalent even in young children and is caused by various genotypes of *O. tsutsugamushi*, including Karp, Kato, Gilliam, and JP genogroups in Myanmar. Moreover, detection of SFTSV infection, as well as potential coinfection with scrub typhus, in the Myanmar patients suggests that SFTSV infection may have a much wider distribution in Eastern and Southern Asia.

MATERIALS AND METHODS

1. Ethics statement

The study in Korea was approved by the Institutional Review Board of Seoul National University Hospital. Study in Myanmar was approved by the ethics review committee of the Department of Medical Research, Ministry of Health and Sports in Myanmar (Ethics/DMR/2018/134), and the institutional review board of Seoul National University Hospital (IRB 1910-057-1069).

2. Subjects and sample collection

I examined 2,328 sera samples of patients from 2000 to 2003 for the diagnosis of rickettsial disease at Seoul National University, Seoul, South Korea. The patients suffered from an acute febrile illness from November 1, 2000, to November 1, 2003. In Myanmar, whole blood samples were collected from clinically suspected scrub typhus patients admitted to local hospitals in Sagaing and Magway provinces between February 2018 and January 2009. Specimens collected from 152 patients were transported to the University of Medicine 1 (Yangon, Myanmar) to prepare sera and stored at -80°C until use in Seoul National University.

3. Antibodies and reagents

Anti-NP (SFTS virus) (Huaiyangshan Virus) (IT-017-006M11)(Immune Technology, New York, USA). Anti-NP antibody produced from Rabbit (Polyclonal antibody production Service in Abclon, South Korea). Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-human IgG secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) were used for immunoblotting and HRP-conjugated anti-mouse IgG1, IgG2c (Santa Cruz Biotechnology) were used for ELISA. The Alexa Fluor 488 or Alexa Fluor 594-conjugated anti-mouse and human antibodies used in the immunofluorescence assays were purchased from Molecular Probes (Invitrogen, Carlsbad, California, USA). For the focus forming assay, methyl cellulose was purchased from Sigma (Madison, Wisconsin, USA) (M0387-100G). HiSenScript TM RH (-) RT-PCR

Pre-Mix was used in Reverse Transcription PCR process, synthesizing cDNA from RNA of SFTSV, followed by amplifying cDNA products by PCR process (AccuPower R Pfu PCR Premix, K-2024, Bioneer, South Korea). To amplify the *tsa56* gene of *O. tsutsugamushi* using a set of primers (forward primer: GATCAAGCTTCCTCAGCCTACTATAATGCC, reverse primer: CTAGGGATCCCGACAGATGCACTATTAGGC). To amplify the *NP* gene of SFTSV using set of primers: forward primer: ATCGTCAAGGCATCAGGGAA, reverse primer: TTCAGCCACTTCACCCGAA).

4. Cell culture

L929 cells (ATCC; NCTC929), Vero cell (ATCC CCl-81) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Daegu, South Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Welgene), 100 U/mL penicillin and 100 U/mL streptomycin (Gibco BRL, Gaithersburg, Maryland, USA) at 37°C in 5% CO_2.

5. Preparation of *Orientia tsutsugamushi* and amplification of nucleic acids

The Boryong, Karp, Kato, and Gilliam strain of *O. tsutsugamushi* were propagated in L929 cell. At 3 to 4 days post-infection, infectivity was determined using an indirect immunofluorescence assay. When an infection rate of >90% was achieved, the cells were harvest by centrifugation at 1000 x g, 10 minutes, 4°C. Infected L929 cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.2), spotted onto spot slides, and fixed with cold acetone for 10 minutes. The slides were stored at -70°C until used.

Total DNA or viral RNA in sera was extracted using DNeasy Blood and Tissue kit or QIAamp viral RNA Mini kit (QIAGEN, Germany), respectively, according to the manufacturer's instruction. For molecular diagnosis of *O. tsutsugamushi*, polymerase chain reaction (PCR) was performed to amplify the *tsa56* gene of *O. tsutsugamushi* using a set of primers (forward primer: GATCAAGCTTCCTCAGCCTACTATAATGCC, reverse primer: CTAGGGATCCCGACAGATGCACTATTAGGC). To detect SFTSV RNA, Reverse transcription PCR (RT-PCR) was performed to amplify the partial small (S) segment of the viral RNA from the patients' sera and confirm SFTSV infection. SFTSV mRNA was extracted from the

patients' sera using TRIzol reagent (Promega, Madison, USA). mRNA was then subjected to reverse transcription using HiSenScript TM RH(−) RT−PCR Pre−mix, synthesizing cDNA from RNA of SFTSV, followed by amplifying cDNA products by PCR process (AccuPower R Pfu PCR Premix, K−2024, Bioneer) to amplify the *NP* gene of SFTSV using a set of *NP* gene−specific primers. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Warrington, UK).

6. TA cloning

Taq DNA polymerase (Bioneer) is used to amplify the DNA fragment in PCR process with a set of primers *tsa56* gene of *O. tsutsugamushi* under the following conditions: denaturation for 10 minutes at 94°C, followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing 30 seconds at 63°C, an extension for 1 minute at 72°C and after 30 cycles, an extension for 7 minutes at 72°C. The DNA ligation step was followed by a manuscript by pGEM®−T Easy Vector System I kit (Promega). 1 μl of ligated using T4 DNA Ligase was transformed into 100 μl *E. coli* (*Escherichia coli*) (TOP10− Cat no. C4040−10, Invitrogen, Waltham, MA USA) cells and incubated on ice for 30 minutes. A 42°C heat−shock of 45 seconds was performed, followed by immediate placement on ice for 10 minutes. 500 μl of LB media (Luria−Bertani (broth)) (Affymetrix/ USA Agar, Waltham, Massachusetts, USA) were added to the bacterial suspension before incubating at 37°C for 1 hour. The entire aliquot was plated out on 1.5% (w/v) LB Agar plates with 100 μg/ml ampicillin and incubated overnight at 37°C. Colonies of *E. coli* were isolated from commercial sources through plating on LB agar (Affymetrix/USA Agar). Single colonies were isolated into 2 ml of relevant media overnight. Extract DNA from *E. coli* culture by LaboPass Plasmid kit Purification Kit (COSMO Genetech, South Korea).

7. Cloning, expression, and purification of proteins.

The synthesized *NP* gene was then cloned into a pET28a vector (Novagen, Gibbstown, New Jersey, USA) for sequence analysis and protein expression. For the expression and purification of NP proteins, the gene

containing pET-28a constructs were transformed into *E. coli* BL21 (DE3) strain (RBC Bioscience Co., Taipei, Taiwan). The strain was grown into LB broth supplemented with 50 μg/ml kanamycin at 37°C overnight. Cultures were inoculated in fresh LB broth and grown at 37°C until the optical density at 600 nm (OD600) reach to 0.5. Protein expression was induced by adding 0.1 mM isopropyl-D-thiogalactoside (IPTG; Duchefa, Zwijndrecht, Netherlands) for 16 hours at 16°C. Bacteria were harvested by centrifugation at 1,000 x g for 10 minutes, resuspended in binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer) containing 1 mg/ml of lysozyme, incubated at 4°C for 30 minutes, and disrupted by sonication on ice for 5 minutes. The sonicated lysates were centrifuged at 1600 x g for 20 minutes at 4°C and the supernatants applied to a Ni-nitrilotriacetic acid (NTA) His-resin (Qiagen). His-tagged proteins bound to the Ni-NTA resin were eluted with elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 250 mM imidazole) and serially dialyzed against elution buffer to remove any free imidazole. The identity and purity of proteins were assessed by Western Blotting and Coomassie Blue staining, respectively.

8. Immunofluorescence microscopy

Immunofluorescence microscopy was used to visualize *O. tsutsugamushi.* After seeding *O. tsutsugamushi* infected L929 cell in the Diagnostic microscope slides and fix, permeabilized by Acetone, within 15 minutes. Incubated with pooled scrub typhus human serum for 1 hour at room temperature, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated mouse anti-rabbit IgG (Invitrogen, Carlsbad, California, USA). Seroconversion IgG observed with immunofluorescence assay using *O. tsutsugamushi* and patients' sera were started from 1:40, 4 fold increase, a serial dilution of patients' sera titer. Images were analyzed and processed using the Olympus Fluoview software (Olympus, Tokyo, Japan).

9. Enzyme-linked immunosorbent assay (ELISA)

To scan the positive patient sample or determine the antibody titers,

immunoassay plates (96-well plates; Nunc, Rochester, New York, USA) were coated with 100 μl of purified antigen at a final concentration of 1 μg/ml at 4°C overnight. The plates were then blocked for 2 hours at room temperature with PBS containing 5% skim milk. 100 μl of serum samples serially diluted in 2-fold were incubated at room temperature for 2 hours. After washing with PBS containing 0.05% Tween 20 (0.05% PBST), 100 μl of 1:10,000 diluted HRP-conjugated mouse anti-human IgM, total IgG (Southern Biotechnology Associates, Birmingham, Alabama. USA) was added and incubated at room temperature for 1 hour. Wells were washed with 0.05% PBST and incubated with 3,3,5,5' -tetramethylbenzidine (TMB) peroxidase substrate solution (KPL, Gaithersburg, Maryland, USA) for 10 minutes. The reactions were stopped by addition of 1 M phosphoric acid (H3PO4) solution. Absorbance was measured at 450 nm using a microplate reader (Beckman Coulter Inc., Fullerton, California, USA). Specific human IgG against NP protein was tested with investigated patients' samples from one fixed dilution factor (1:100) of serum. The x-axis indicates the number of patient serum, and the y-axis indicates the optical density (O.D.) at 450 nm. The cutoff values are calculated following the formula of the form "mean + 3 standard deviation of negative controls"

10. Focus forming assay

SFTSV stock was diluted in 10-fold increments, and each dilution (200 μl) was inoculated onto a monolayer of Vero cells in 24-well plates. After incubating for 1 hour at 37°C, the suspension was removed from each well, and each well was over-aid with 1 ml of 1% methyl cellulose solution. The cells were cultivated for 7 days in a 37°C incubator with 5% CO2. The methyl cellulose overlay was removed from each well, and the monolayers were fixed with methanol-acetone (1:1) (1 ml) for 30 minutes. After washing 3 times (15 minutes/time) with phosphate-buffered saline (PBS), the monolayers were blocked with 3% Bovine Serum Albumins in PBS for 1 hour and were incubated for 1 hour with an anti-NP antibody against SFTSV or patient serum with serial dilution factor from 1:10 to 1:640. After washing 3 times with PBS, the monolayers were incubated for 1 hour with 1:500 Alexa Fluor

594-conjugated anti-Human and Alexa Fluor 488-conjugated anti-rabbit antibodies used in the immunofluorescence assays were purchased from Molecular Probes (Invitrogen, Carlsbad, California, USA). Images were analyzed and processed using the Olympus Fluoview software.

11. Immunochromatography test (ICT)

Immunochromatography test (ICT) strips containing TSA56 and ScaA antigens from *O. tsutsugamushi* Boryong, Gilliam, and Karp genotypes were manufactured (Bore Da Biotech Co., Seongnam, South Korea) and tested for rapid diagnosis of scrub typhus according to the manufacturer's instruction. Results were visualized in 15 minutes after loading mixtures of a chasing buffer (200 μl) containing gold particles and 50 μl of patient serum on a sample pad. Representative images of ICT results detect specific IgG against TSA56 and ScaA antigen in suspected scrub typhus patients' sera. The red band appearing on the control line (C) and test line (T) including TSA56 and ScaA line concurrently or either way was regarded as positive. The test was considered as negative when only the control band appeared as red. And if no band appeared on the control line, then the test was considered invalid.

12. Sequence analysis

Severe fever with thrombocytopenia syndrome virus is isolated from patient serum (GenBank accession no. MN329148.1). Sequence information for *NP* genes was found in the genomes of SFTSV strains (GenBank accession no. KP663733.1). Genetic and phylogenetic analyses were conducted by aligning published sequencing of SFTSV obtained from China, Japan, and South Korea, Vietnam. In order to estimate and compare the degree of genetic diversity of *tsa56* genes with other *O. tsutsugamushi* genes, I collected 17 bacterial genes including the *tsa56* genes ([1] reference) from two complete genome sequences (Boryong, and Ikeda strain and seven draft genomic contigs (strain Bio project accession no; Gilliam: PRJNA212442, Karp: PRJNA212456, Kato: PRJNA212440, TA716: PRJNA212457, TA763: PRJNA212454) available in Bioproject (http://www.ncbi.nlm.nih.gov/bioproject).

Multiple sequence alignment was performed using the ClustalW algorithm in

MEGA version 6.0. Phylogenetic analyses were performed based on the complete sequence of the S segment of SFTSV using the maximum likelihood (ML) method based on Kimura's 2-parameter model. The reliability of the ML tree was evaluated by the bootstrap treat with 1,000 replication.

RESULTS

1. Severe fever with thrombocytopenia syndrome virus jnfection or mixed jnfection with scrub typhus in South Korea 2000-2003

I examined 2,328 sera samples collected from patients who suffered from an acute febrile illness presenting from November 1, 2000, to November 1, 2003, for the diagnosis of Rickettsia disease at Seoul National University, South Korea.

1.1. Analysis of SFTSV NP-specific antibody response by ELISA method

2,328 samples were tested for IgM and IgG antibodies against SFTSV-specific NP protein by ELISA method. There were no samples in which SFTSV NP-specific IgM antibodies could be detected. SFTSV NP-specific IgG antibodies were detected in the sera of 37 patients (37/2,328, 1.6%) (Figure 1). In the year 2000, there were 8 samples (8/400, 2.0%) positive for the SFTSV NP-specific IgG antibody. In the year 2001, among 188 tested samples, 3 samples (3/188, 1.5%) were positive for the SFTSV NP-specific antibody. In the year 2002, 12 samples (12/441, 2.7%) detected the SFTSV NP-specific antibody. In the year 2003, there were 14 patients' sera (14/1,299, 1.1%) which tested positive for the SFTSV NP-specific antibody response (Figure 1) (Table 2).

1.2. Baseline characteristics of patients positive with IgG against SFTSV-specific NP protein

In 37 patients' sera positive for the SFTSV NP-specific antibody response via the ELISA method, the mean age was 61.07±14.1 years. Both groups aged from 21 to 30 years old and 31 to 40 years old had 1 patient (1/37, 2.7%). 5 patients (5/37, 13.5%) were aged 41 to 50 years old. The group aged 51 to 60 years old had 7 patients (7/37, 18.9%). The over 60 year-old group included the highest number of patients at 14 patients (14/37, 37.8%) (Table 1) (Figure 2). The 37 tested patients included 10 males (10/37, 37.03%) and 27 females (27/37, 72.97%). 9 samples (9/37, 24.0%) had no information about the age and gender (Table 1) (Figure 2). The seasonal distribution of 37 patients' sera positive for the SFTSV NP-specific IgG antibody was obvious. The month

with the highest number of patients testing positive for the SFTSV NP-specific antibody was November with 25 samples (25/37, 67.5%). In October and December, there were 5 positive samples (5/37, 13.5%) and 3 positive samples (3/37, 8.1%), respectively. The remaining months have 1 positive sample or no positive samples (Table 1) (Figure 2). In hospital records, by IFA method, some samples were positive with Hantavirus which caused Hemorrhagic fever and renal syndrome (HFRS), *Leptospira* which caused Leptospirosis, and *R. typhi* which caused murine typhus. Among the 37 tested samples, one sample tested positive for *Leptospira* and 4 samples tested positive for *R. typhi* (Table 1). Two samples (2/37, 5.4%) tested positive for SFTSV and *R. typhi* (≥1:1,280) in 2001. There was 1 sample (1/37, 2.7%) that tested positive for SFTSV and *O. tsutsugamushi* (≥1:2,560) and *Leptospira* (≥1:160), and 1 sample (1/37, 2.7%) that tested positive for SFTSV and *O. tsutsugamushi* (≥1:2,560) and *R. typhi* (≥1:160) in 2002. There was 1 sample (1/37, 2.7%) that tested positive for SFTSV and *R. typhi* (≥1:80) in 2002.

1.3. Detect SFTSV-specific IgG antibody in patients' sera

All samples positive for the SFTSV NP-specific IgG antibody via the ELISA method were confirmed through staining SFTSV plaque by immunofluorescence assay (IFA). Among the 37 samples, SFTSV NP-specific IgG antibodies were detected in the sera of 15 samples (15/37, 40.5%). In the year 2000, among the 8 samples positive for the SFTSV NP-specific IgG antibody via the ELISA method, there were 6 samples (6/8, 75.0%) that tested positive via the IFA method. In the year 2001, no samples could detect the SFTSV NP-specific IgG antibody. Otherwise, there were 2 samples (2/3, 66.6%) that tested positive for *R. typhi* via the IFA method. In the year 2002, 3 out of the 12 patients' sera (3/12, 25.0%) that tested positive via the ELISA method were detected with the SFTSV NP-specific IgG antibody. Among the 12 tested samples in 2002, 1 sample (1/12, 8.3%) and 2 samples (2/12, 16.6%) were positive for the *Leptospira*-specific antibody and *R. typhi*-specific antibody, respectively via IFA. In the year 2003, 6 of the 14 samples (6/14, 42.9%) carried titers (≥1:160) of IgG antibodies specific to

SFTSV, as measured via IFA (Figure 3) (Table 2).

1.4. Detection of SFTSV-specific *NP* gene by RT-PCR from the patients' sera and the phylogenetic tree constructed based on partial S segment sequences of SFTSV

To detect the SFTSV-specific *NP* gene by RT-PCR from the patients' sera, I screened 37 patients' whose sera was positive for the SFTSV NP-specific antibody response in South Korea from 2000-2003. The SFTSV-specific *NP* gene was detected in the sera of 9 samples (9/37, 24.3%) among the 37 positive samples from the ELISA method (Figure 4). In the year 2000, 2 samples (2/8, 25.0%) were clearly positive for the SFTSV-specific *NP* gene RNA. In the year 2001, 1 out of the 3 patients' sera (1/3, 33.3%) that tested positive from the ELISA method was detected with the SFTSV-specific *NP* gene. In the year 2002, 12 patients' sera were positive with IgG antibodies against SFTSV via ELISA. Among them, there were 5 samples (5/12, 41.7%) which detected the SFTSV-specific *NP* gene. In the year 2003, 6 samples (6/14, 42.9%) were clearly positive for the SFTSV-specific *NP* gene RNA. The percentage of patients' sera which detected the SFTSV-specific *NP* gene had rapidly increased each year from the years 2000 to 2003. The percentage of positive samples in the year 2000 was 25.0%. In the year 2001, the percentage of positive samples was up to 33.3% of patients. This percentage in the years 2002 and 2003 dramatically increased to 41.7% and 42.9%, respectively (Table 2). The partial S segment sequences of SFTSV were detected in 9 patients (9/37, 24.3%) via two rounds of RT-PCR, and the resulting phylogenetic tree of the partial S segment compared to 19 proto-genotypes showed that the partial S segment's isolates were similar to those from China, South Korea, and Japan. Among the 9 positive samples, there were 5 isolated samples which differed by only one base from the other 4 isolates, suggesting genetic homogeneity of SFTSV in South Korea (Figure 5).

1.5. The presence of antibody response specific for *O. tsutsugamushi* in patients' sera

37 patients' sera positive for the SFTSV NP-specific IgG antibody via ELISA were tested for IgG antibodies against *O. tsutsugamushi* using IFA. *O. tsutsugamushi*-specific IgG antibodies were detected in the sera of 17 patients (17/37, 45.9%) (Figure 6) (Table 2). In the year 2000, there were 4 samples (4/8, 50%) that tested positive for the *O. tsutsugamushi*-specific IgG antibody via IFA. In the year 2001, among 3 samples tested, 1 sample (1/3, 33.3%) was confirmed for the presence of the *O. tsutsugamushi*-specific IgG antibody in the patients' sera. In the years 2002 and 2003, there were 4 samples (4/12, 33.3%) and 8 samples (8/14, 66.7%) in which antibody response specific to *O. tsutsugamushi* was detected, respectively. Among all patients' sera positive for the SFTSV NP-specific antibody via the ELISA method, 45.9% of samples detected the *O. tsutsugamushi*-specific IgG antibody. Sometimes patients had a primary infection, the initial infection caused by one pathogen such as SFTSV or *O. tsutsugamushi* which can continue with a secondary infection by a different pathogen (SFTSV or *O. tsutsugamushi*). It was considered as an evidence for potential coinfection with the two pathogens *O. tsutsugamushi* and SFTSV.

1.6. Amplification of *O. tsutsugamushi*-specific *tsa56* gene by PCR from the patients' sera and the phylogenetic tree constructed based on the *O. tsutsugamushi*-specific *tsa56* gene sequences

For the molecular diagnosis of scrub typhus, all of the ELISA samples positive for SFTSV were examined by PCR to confirm the *O. tsutsugamushi* infection and identify the genotypes of *O. tsutsugamushi* in South Korea from November 1, 2000, to November 1, 2003. By PCR process, the *O. tsutsugamushi*-specific *tsa56* gene was detected in 14 patients (Figure 7). Among these 14 patients, 12 patients belonged to the group aged 50 and over, only 1 patient was 47 years old, and 2 patients lacked information about age. After direct DNA sequencing of PCR products, among the samples 14 were detected to have the *tsa56* gene. Nine samples showed a clear genotype of *O. tsutsugamushi* with the 3 genotypes Karp_C (6/37, 16.2%), Boryong (1/37, 2.7%), Gilliam (2/37, 5.4%). Five samples showed a mixed-infected *O. tsutsugamushi* strain (5/37, 13.5%). By TA cloning, I was only capable of classifying strains

isolated from the mixed-infected *O. tsutsugamushi* in 2 samples, both of which were mixed between the Karp and Gilliam strains (Figure 8). Three mixed samples could not be classified by *O. tsutsugamushi* genotypes. Phylogenetic analysis of the 15 samples detected the *tsa56* gene by comparison with the sequences from 16 proto-genotypes which revealed Boryong (1/37, 2.7%), Karp_C (8/37, 21.6%) and Gilliam (4/37, 10.8%) (Figure 8). The surveillance for the 37 positive samples from South Korea from 2000-2003 showed that Karp_C (21.6%) is more abundant compared to the other detected genotypes Boryong (2.7%) and Gilliam (10.8%).

1.7. Potential coinfection with *O. tsutsugamushi* and SFTSV

Based on the results of the molecular detection of the two pathogens *O. tsutsugamushi* and SFTSV, there were 4 samples (4/37, 10.8%) which detected both the presence of the *O. tsutsugamushi*-specific *tsa56* gene and SFTSV-specific *NP* gene, considering the potential coinfection with *O. tsutsugamushi* and severe fever with thrombocytopenia syndrome virus (SFTSV) (Table 3). One sample was collected from the year 2000 and three samples were collected from the year 2003. Four samples showed potential coinfection with 2 kinds of the pathogen which were all classified under 2 genotypes of *O. tsutsugamushi* Karp and Gilliam. Among 37 patients' sera positive with an SFTSV NP-specific antibody response, 15 patients' sera (15/37, 40.5%) showed a positive result by staining an SFTSV plaque (≥1:160). Five samples (5/37, 13.5%) were positive for both pathogens *O. tsutsugamushi* and SFTSV via IFA method with titer ≥1:1,280 and ≥1:160, respectively (Table 3). Two samples were collected from 2000, 1 sample was collected from 2002, and the remaining sample was collected from 2003. There were 5 samples (5/37, 13.5%) which showed potential coinfection between 2 pathogens by detecting the SFTSV-specific *NP* gene and *O. tsutsugamushi*-specific IgG antibody. One sample tested positive for the *O. tsutsugamushi*-specific IgG antibody with titer ≥1:1,280 and was collected in 2003. Four remaining samples were collected in 2003 with antibody titer ≥1:80 specific for *O. tsutsugamushi*. Among the 37 samples, 4 samples (6/37, 16.2%) were positive for the SFTSV-specific antibody and which also detected the *O. tsutsugamushi*-specific *tsa56* gene. One sample with potential coinfection

with *O. tsutsugamushi* and SFTSV was collected in the year 2000, 1 sample was from the year 2002, and the 4 remaining samples were all collected in the year 2003.

2. Genotype diversity of *O. tsutsugamushi* in scrub typhus patients and coinfection with severe fever with thrombocytopenia syndrome virus in Myanmar

I analyzed 152 sera collected from suspected scrub typhus patients admitted to local hospitals in the Sagaing and Magway provinces, located in the central part of Myanmar (Figure 9). There were 129 patients' sera from Sagaing (129/152, 84.8%), and the 23 remaining samples were collected from Magway (23/152, 15.2%). This study was conducted in two phases. In phase 1, all patients fulfilling the inclusion of the criteria patients of acute undifferentiated fever (AUF) were recruited. This formed the sampling frame for identifying study participants for this study. Phase 2 of the study wherein all patients of AUF which were found positive for the *O. tsutsugamushi* and SFTSV pathogen were included for further evaluation.

2.1. Baseline characteristics and the results of serological and molecular diagnosis of patients' samples in Myanmar

The baseline characteristics of the patients were summarized in Table 4. All of the patients with acute undifferentiated fever were successfully treated in the local clinics and recovered from febrile illness. The mean age of the suspected scrub typhus patients was 27±19.8 years old and ranged from 2 to 73 years old. The standard deviation of the suspected patients is 19.8, which is large, showing the large variety of suspected patients' ages. Scrub typhus disease was suspected the most in patients who were under 10 years old (38/152, 25.0%), and from 11 to 20 years old with a fever duration of 4.57±1.5 days and 5.8±2.6 days, respectively. There were 23 patients aged from 21 to 30 years old (23/152, 15.1%) with a fever duration of 6.1±2 days. Ten patients (10/152, 6.6%) were aged 31 to 40 years old, and patients over 61 years old, had a fever duration of 5.8±2.7 days, and 9.9±4.8 days, respectively.

There were 17 patients aged 41 to 50 years old (17/152, 11.2%) with a fever duration of 7.4±2.8 days. The patient population aged 51 to 60 of 16 people (16/152, 10.5%) with a fever duration of 6.6±3.12 days. One patient had no information about the fever duration (Table 4) (Figure 10). 93 suspected patients (93/152, 61.2%) were male, almost double that of female patients. The male group had a fever duration of 6±2.9 days, and the female group had a fever duration of 6.2±2.9 days. Eschar was observed in 144 patients (144/152, 94.7%) and the mean fever duration was 6 days (S.D.:±2.9 days), Nine patients (9/152, 5.9%) could not observe eschar, 3 of which were under 10 years old, 3 of which were from 10 to 20 years old, and 3 of which were 20 to 50 years old. 25 suspected patients (25/152, 16.4%) had muscle and joint pain (myalgia) with the mean age being 36.4±14.7 years. Six patients (6/152, 3.9%) lacked information about myalgia symptoms. The rash symptom was observed in 3 patients (3/152, 2.0%) from 20 to 40 years old. Among 3 patients with a rash, 2 suspected patients also had eschar, and 1 suspected patient had myalgia and rash but no eschar. There were 8 patients (8/152, 5.26%) only having fever but could not observed as having eschar, myalgia, and rash with a fever duration of 7±1.5 days (Table 4).

2.2. Diagnosis test specific for SFTSV in 152 patients' sera and the phylogenetic tree constructed based on partial S segment sequences of SFTSV

Among the 152 patients' sera, samples positive with the SFTSV-specific antibody were confirmed by staining SFTSV plaque with immunofluorescence assay (IFA). There was only 1 patient's serum (1/152, 0.65%) which tested positive for a SFTSV-specific antibody response (≥1:40) with a fever duration of 8 days, and a mean age of 33 years old (Figure 12). The positive patient had both eschar and myalgia (Table 5). Finally, I investigated for potential infection with SFTSV in Myanmar patients via RT-PCR analysis. Among the 152 patients' sera, 5 sera (5/152, 3.3%) were positive for the partial S segment of the SFTSV RNA genome, indicating SFTSV infection. It is also interesting to note that 4 out of the 5 positive patients had eschar and 4 of the 5 patients were

under 15 years of age (Table 5). A phylogenetic analysis of the partial S segment sequences compared with 40 proto-genotypes showed that 4 isolates were the same as those previously reported from Vietnam, and one isolate differed by one base from the other 4 isolates (Figure 13), suggesting genetic homogeneity of SFTSV in Southern Asia.

2.3. Initial serological diagnosis of patients' sera in 152 samples in an immunochromatography test (ICT)

The initial serological diagnosis of 128 sera using immunochromatography test (ICT) strips coated with TSA56 revealed 36 patients' sera (36/128, 28.1%) testing positive for the *O. tsutsugamushi* TSA56-specific IgG antibody, with a fever duration of 6.7±3.35 days. ICT strips coated with ScaA have revealed 25 patients' sera (25/128, 19.5%) positive for the *O. tsutsugamushi* ScaA-specific IgG antibody, with a fever duration of 5.8±2.8 days. 20 patients' sera (20/128, 15.6%) reacted with both antigens TSA56 and ScaA specific for *O. tsutusgamushi* (Figure 14) (Table 5). Among all of the positive patients' sera from the ICT strips, there were no samples in which *tsa56* gene sequences could be detected. Moreover, the overall positive rate of the home-made ICT was relatively low (41/128, 32%) when compared to the IFA detecting IgG responses against the bacterial antigen (101/128, 78.9%).

2.4. The presence of antibody response specific for *O. tsutsugamushi* in patients' sera

To confirm serological possibility against the bacterial antigen, I also conducted IFA using cells infected with *O. tsutsugamushi*, the gold standard method for diagnosis of scrub typhus (Figure 15). Among 152 patients' sera, 119 (119/152. 78.3%) samples were positive (≥1:40) for the *O. tsutsugamushi*-specific IgG antibody, with a fever duration of 6.2±3.2 days, and 90 samples (90/152, 59.2%) were positive for the *O. tsutsugamushi*-specific IgM antibody, with a fever duration of 6.4±3.2 days (Table 5). There were 69 patients' sera (69/152, 45.3%) which were positive for both the *O. tsutsugamushi*-specific IgG and IgM antibodies. Median titers of the positive sera were 1:640 for both the IgG

and IgM antibodies (Figure 15) (Table 5). 13 patients ' sera (13/152, 8.6%) among the suspected scrub typhus samples were negative for both the IgG and IgM antibodies against the bacterial pathogen. Eschar was observed in all 13 of these samples. In the case of suspected scrub typhus patients with no eschar, 3 patients ' sera (3/152, 1.97%) were positive for both specific IgG (≥1:40) and IgM (≥1:160), 6 samples were positive for specific IgG (≥1:160), and 1 sample was positive for specific IgM (1:160) (Figure 15).

2.5. Amplification of *O. tsutsugamushi*-specific *tsa56* gene by PCR from the patients' sera and the phylogenetic tree constructed based on the *O. tsutsugamushi*-specific *tsa56* gene sequences

For the molecular diagnosis of scrub typhus, all of the samples were examined by PCR to confirm infection and identify the genotypes of *O. tsutsugamushi* in the Myanmar patients. Among the 152 sera, I could detect specific PCR products from 9 *tsa56* gene sequences by a comparison with sequences from 17 proto-genotypes which revealed the presence of at least five genotypes: Karp A (4/9, 44.4%), Karp B (1/9, 11.1%), Kato B (2/9, 22.2%), Gilliam (1/9, 11.1%), and JG_C (1/9, 11.1%) in the Myanmar patients (Figure 16). The genotype *O. tsutsugamushi* in the Myanmar study is more variable than in the previous South Korea study, and Karp_A was more abundant at 44.4%. I investigated all 152 patients' sera by checking for the *O. tsutsugamushi* specific *tsa56* gene sequence. There was 1 sample (1/152, 0.65%) in which I detected the *O. tsutsugamushi*-specific *tsa56* gene with a fever duration of 5 days, although this sample was negative for the *O. tsutsugamushi*-specific IgG antibody response in IFA method, but this patient had an eschar. In 119 samples positive for the *O. tsutsugamushi* specific IgG antibody via IFA, Five samples were detected to have the *O. tsutsugamushi*-specific *tsa56* gene (≥1:160). In the case of IgM, among the 90 positive patients' sera (≥1:40), there were 8 samples which were detected to have the *O. tsutusgamushi*-specific *tsa56* gene.

2.6. Potential coinfection with *O. tsutsugamushi* and SFTSV

Based on the results of molecular and serological detection, 5 sera (5/152,

3.3%) were positive for the partial S segment of the SFTSV RNA genome, indicating SFTSV infection. Furthermore, 3 of the 5 carried high titers (≥1:2,560) of IgG and/or IgM antibodies specific to *O. tsutsugamushi*, as measured via IFA (Figure 15), suggesting coinfection with scrub typhus. All SFTSV-positive patients suffered with a fever for 5 to 7 days and recovered fully from febrile illness. There was 1 suspected patients' serum (No.132 patient) having the SFTSV-specific IgG antibody. From the IFA method, this patients' serum showed a positive result in staining the SFTSV plaque (≥1:320). Patient No.132 had eschar and muscle and joint pain symptoms. Moreover, this sample was positive for the *O. tsutsugamushi*-specific IgM antibody (≥1:40) via IFA method (Figure 15) (Table 6).

Table 1. Summary of the characteristics of patients' sera positive with SFTSV-specific NP protein in South Korea 2000-2003

Age (year), mean ± SD	61.07 ± 14.1
Age distribution, *n* (%)	
NT	9 (9/37, 24.0%)
21 ~ 30	1 (1/37, 2.7%)
31 ~ 40	1 (1/37, 2.7%)
41 ~ 50	5 (5/37, 13.5%)
51 ~ 60	7 (7/37, 18.9%)
61 ~	14 (14/37, 37.8%)
Male/Female, *n* (Male %)	10/27 (37.03%)
Coinfection with other pathogen	3 (3/37, 8.0%)
Hemorrharic fever with renal syndrome	0 (0/37, 0%)
Leptospirosis	1 (1/37, 2.7%)
Murine typhus	4 (4/37, 10.8%)
Year	
2000 year	8 (8/37, 21.6%)
2001 year	3 (3/37, 8.1%)
2002 year	12 (12/37, 32.4%)
2003 year	14 (14/37, 37.8%)
Month	
Jan	0 (0/37, 0%)
Feb	0 (0/37, 0%)
Mar	1 (1/37, 2.7%)
Apr	0 (0/37, 0%)
May	1 (1/37, 2.7%)
Jun	1 (1/37, 2.7%)
Jul	0 (0/37, 0%)
Aug	1 (1/37, 2.7%)
Sep	0 (0/37, 0%)
Oct	5 (5/37, 13.5%)
Nov	25 (25/37, 67.5%)
Dec	3 (3/37, 8.1%)

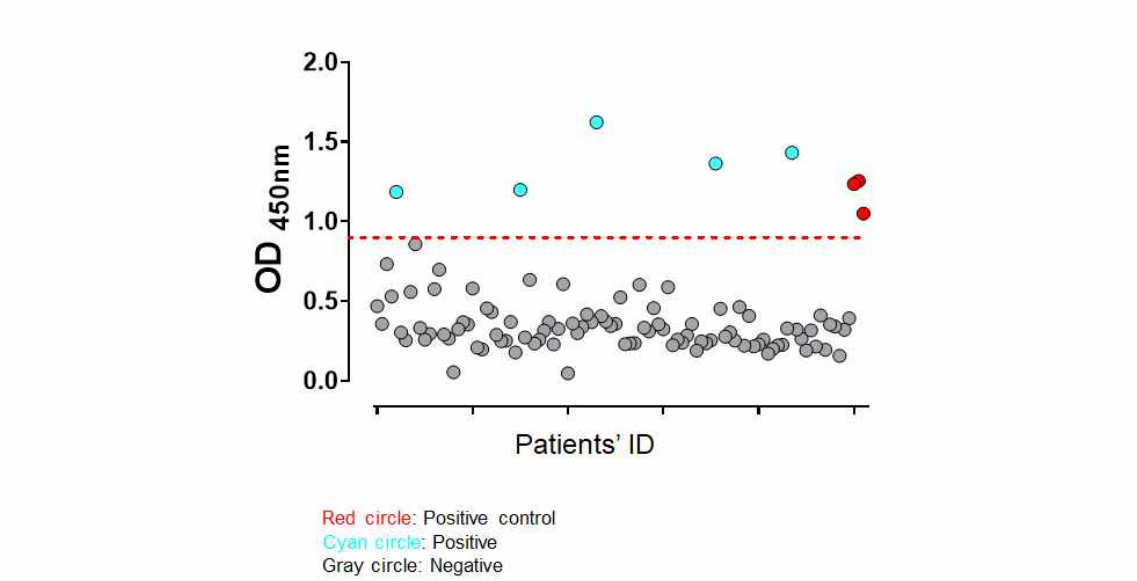

Figure 1. Analysis of SFTSV NP-specific antibody responses in sera collected in South Korea during 2000-2003. The x-axis indicates the IDs of patient sera, and the y-axis indicates the optical density (O.D.) at 450 nm. The figure shows some represented data in the year 2000 using the ELISA method. Positive samples and negative samples were shown in a cyan circle, gray circle, respectively. Positive controls from SFTSV infected patients' sera were drawn in red color. Cut-off titers was determined by using confirmed negative sera and indicated as red dashed line.

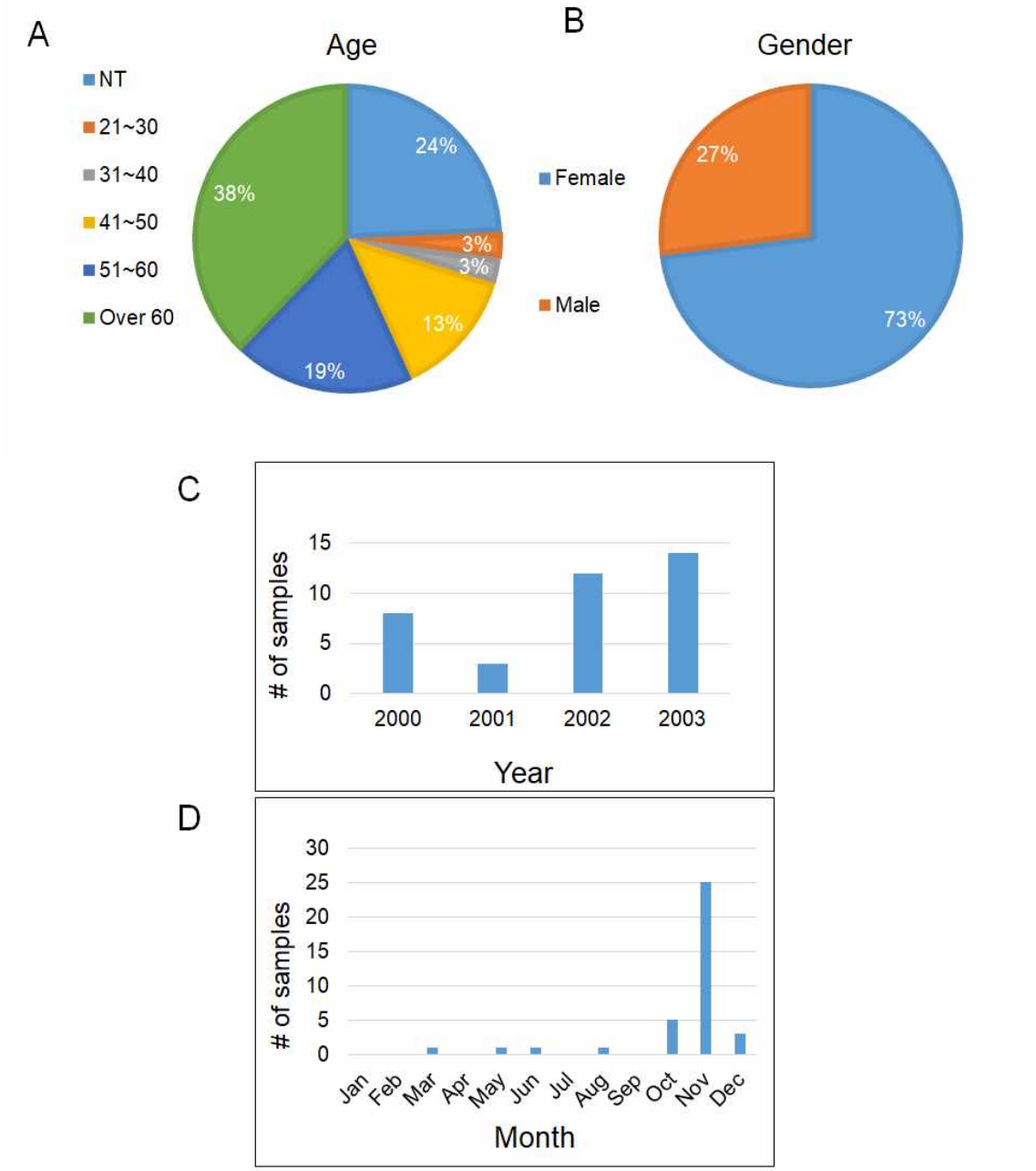

Figure 2. Baseline characteristics of patients with positive IgG against SFTSV NP in South Korea 2000–2003. (A) Patient age distribution, (B) Patient gender distribution, (C) and (D) Timing of sample collection: Year and month, respectively.

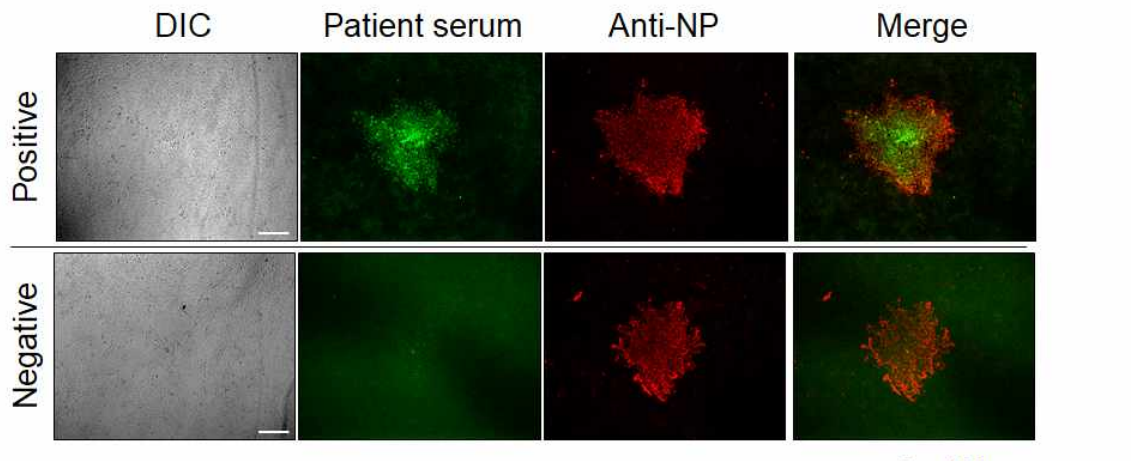

Figure 3. Immunofluorescence assay (IFA) to detect SFTSV-specific IgG antibody responses in patients' sera collected in South Korea during 2000-2003. SFTSV plaque was stained with the patients' sera with a serial dilution of the patients' sera titer and rabbit anti-SFTSV NP antibody as an internal positive control. The upper image is a positive patients' serum that presented the presence of SFTSV-specific IgG antibody response. The lower one is a negative sample that showed the absence of the presence of SFTSV-specific IgG antibody response. SFTSV plaque is stained by patients' serum shown by green color, SFTSV plaque is stained by a rabbit anti-SFTSV NP antibody shown in red color. DIC: Differential interference contrast. Bar: 100μm.

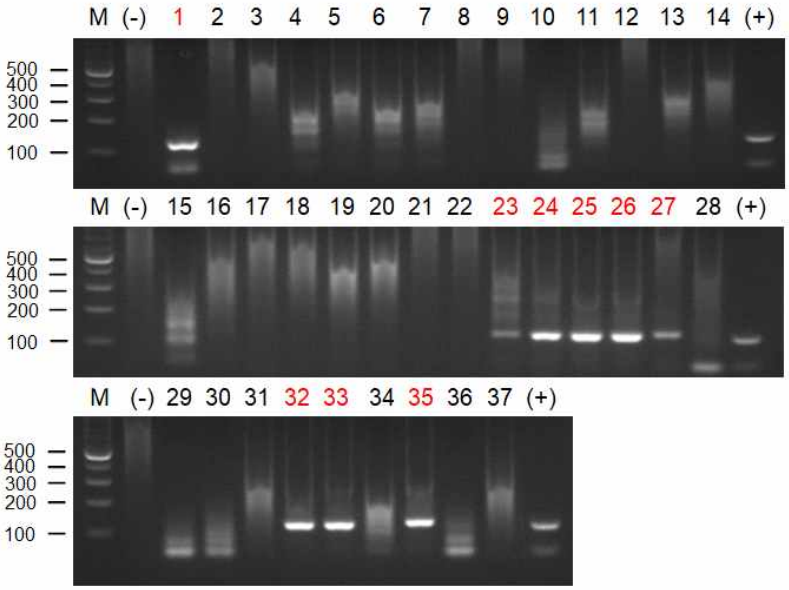

Figure 4. Detection of SFTSV specific *NP* gene (123bp) by RT-PCR from the patients' sera collected in South Korea during 2000-2003. Lane M, 100bp DNA ladder marker; lane (-) and (+), negative and positive control, respectively; Lane 1 to 6, patients' sera in 2000 year; Lane 7 to land 9, patients' sera in 2001 year; Lane 10 to land 19, patients' sera in 2002 year; Lane 20 to land 37, patients' sera in 2003 year. All these patients' sera were positive with SFTSV NP-specific ELISA.

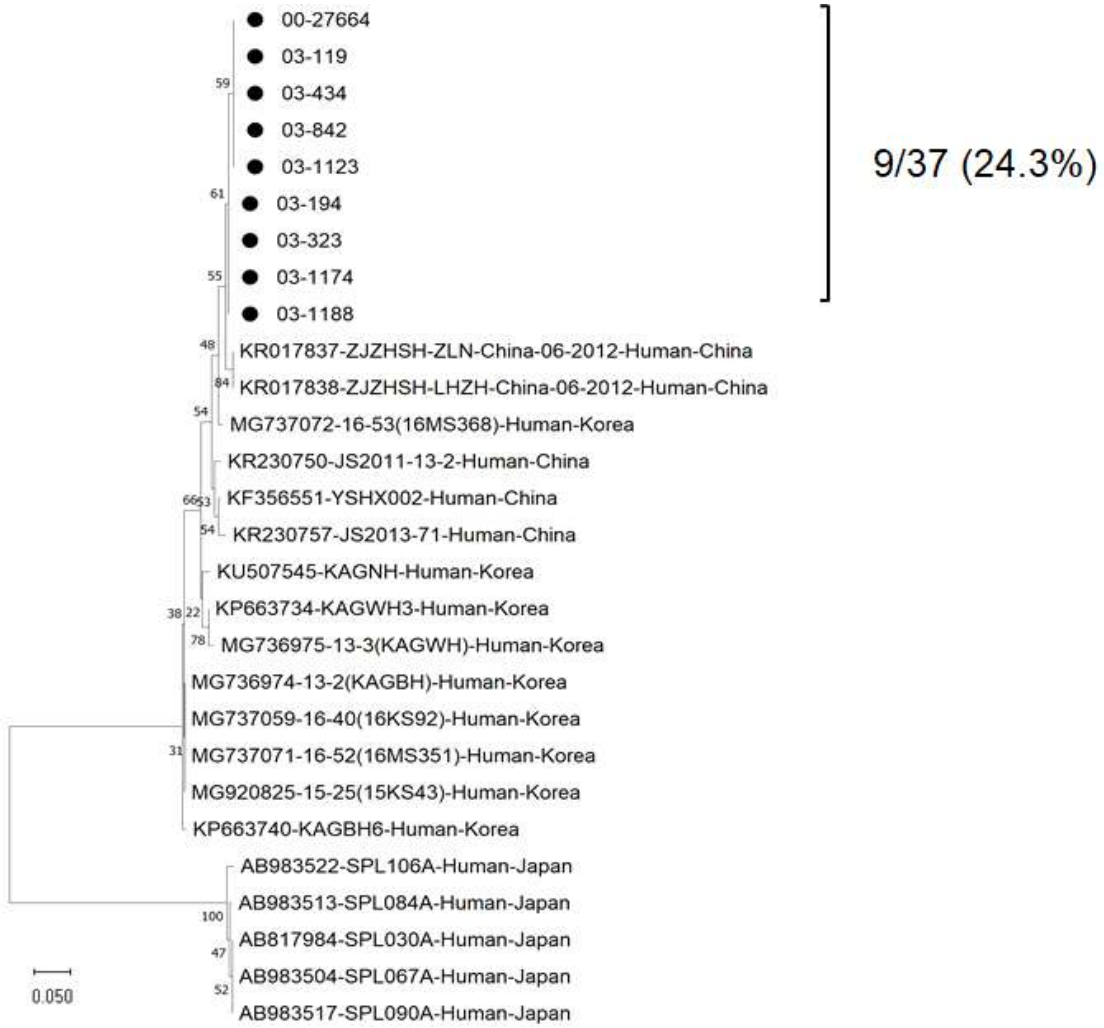

Figure 5. Phylogenetic tree of partial S (NP) sequences of SFTSV in South Korea. The partial S sequences from the stored sera collected from November 1, 2000, to November 1, 2003, that were analyzed in this study are shown in black circle compared with 19 representative genotype sequences. The partial S sequence data for the viruses identified in China, South Korea , and Japan were obtained from NCBI/BLAST.

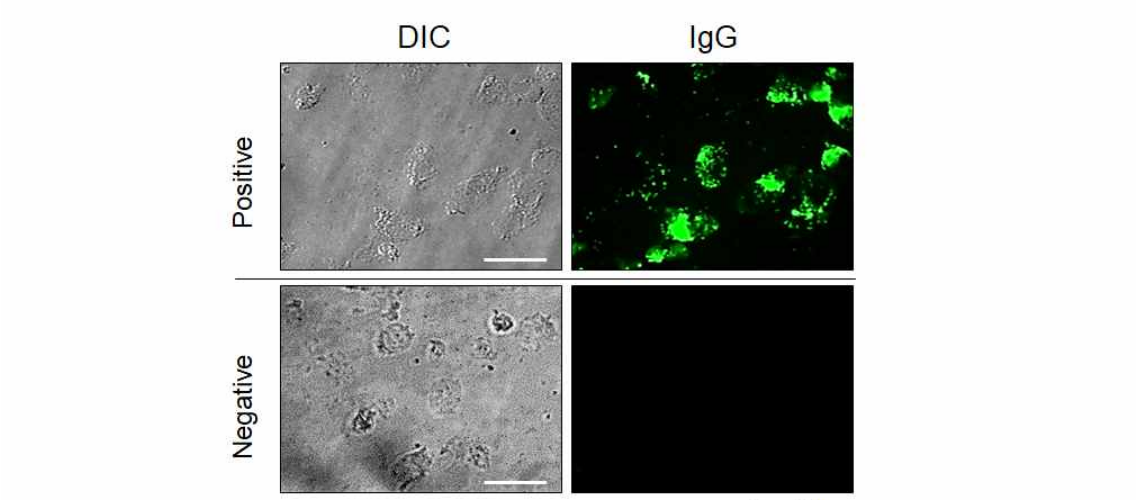

Figure 6. Antibody responses specific to *O. tsutsugamushi* in patients' sera collected in South Korea during 2000–2003. The upper image is a positive patients' serum that presented an antibody specific for *O. tsutsugamushi*. The lower one is a negative sample that showed the absence of an antibody specific for *O. tsutsugamushi.* DIC: Differential interference contrast. *O. tsutsugamushi* (green color). Bar: 20μm.

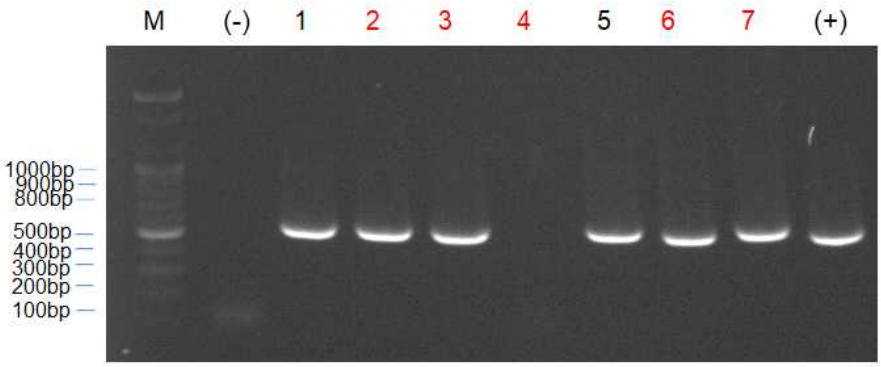

Figure 7. Detection of *O. tsutsugamushi*-specific *tsa56* gene (483bp) by PCR from the patients' sera collected in South Korea during 2000-2003. Lane M, 100 bp DNA marker; lane (-) and lane (+), negative and positive control, respectively. All these patients' sera were positive with SFTSV NP-specific ELISA. The figure shows some represented data using the PCR method.

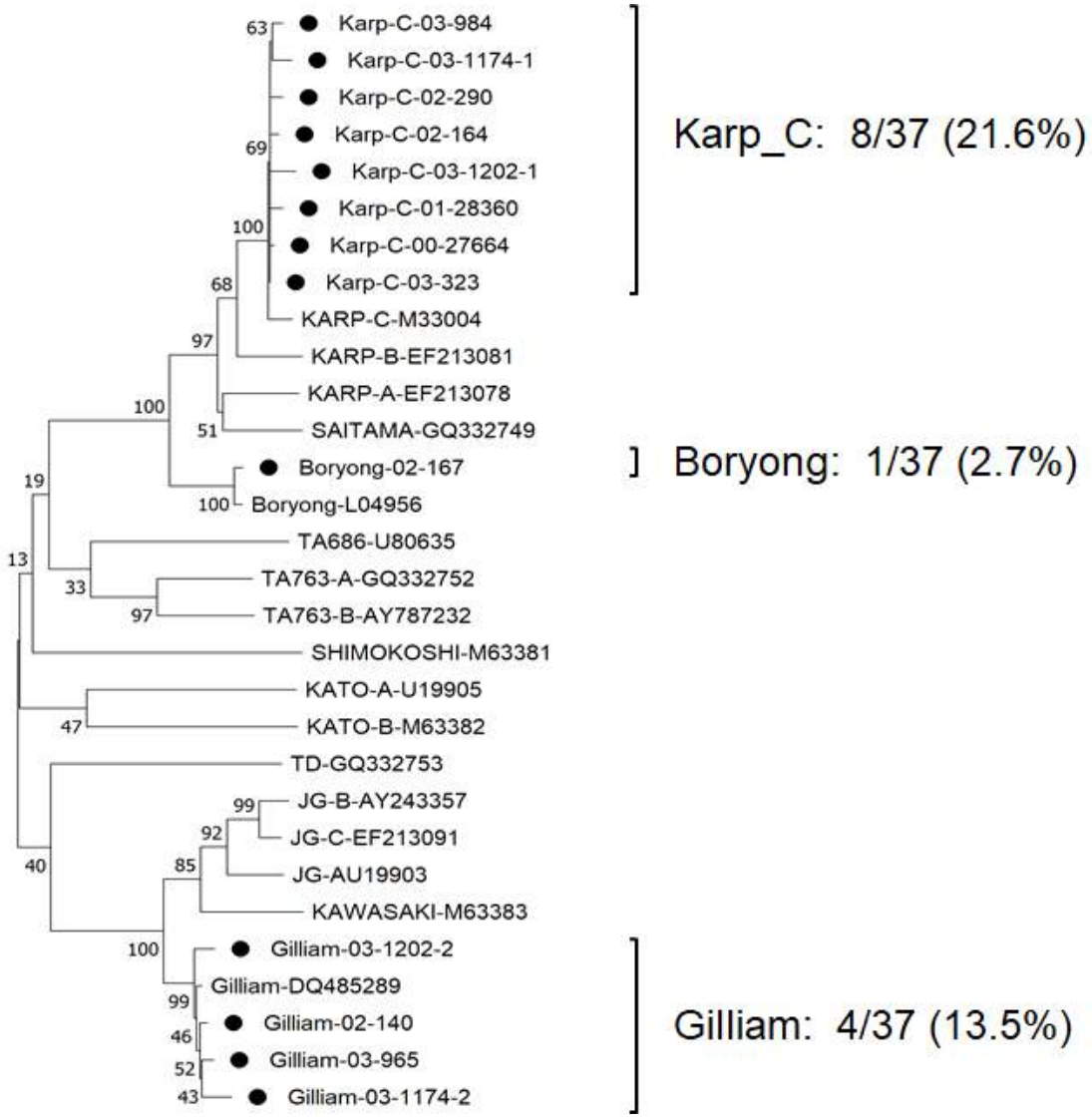

Figure 8. Phylogenetic tree of *O. tsutsugamushi*-specific *tsa56* gene sequences in South Korea. The tree was constructed using the maximum likelihood method with MEGA6. The *tsa56* gene sequences identified in this study are indicated by black circles and compared with 17 representative genotype sequences. The *O. tsutsugamushi tsa56* gene sequence data identified from China, South Korea, Japan, and India were obtained from NCBI/BLAST.

Table 2. Summary of the diagnosis results for tested patients' sera collected in South Korea 2000-2003

Pathogen	Method	2000 year	2001 year	2002 year	2003 year	# of positive samples
SFTSV	ELISA	8/400 (2.0%)	3/188 (1.5%)	12/441 (2.7%)	14/1299 (1.1%)	37/2328 (1.6%)
	RT-PCR	2/8 (25.0%)	1/3 (33.3%)	5/12 (41.7%)	6/14 (42.9%)	9/37(24.3%)
	IFA	6/8 (75.0%)	0/3 (0.0%)	3/12 (25.0%)	6/14 (42.9%)	15/37 (40.5%)
O. tsutsugamushi	IFA	4/8 (50.0%)	1/3 (33.3%)	4/12 (33.3%)	8/14 (66.7%)	17/37 (45.9%)
	PCR	2/8 (25.0%)	1/3 (33.3%)	4/12 (33.3%)	7/14 (50.0%)	14/37 (37.8%)

Table 3. Potential coinfection with *O. tsutsugamushi* and SFTSV in South Korea 2000-2003

Diagnosis criteria	# of positive samples
SFTS (RT-PCR +)/ scrub typhus (PCR +)	4/37 (10.8%)
SFTS (IFA +)/ scrub typhus (IFA +)	5/37 (13.5%)
SFTS (RT-PCR +)/ scrub typhus (IFA +)	5/37 (13.5%)
SFTS (IFA +)/ scrub typhus (PCR +)	6/37 (16.2%)

Table 4. Summary of the characteristics of patients with potential scrub typhus in Myanmar

Age (year), mean ± SD	27.0 ± 19.8
Age distribution, *n* (%)	
~ 10	38 (38/152, 25.0%)
11 ~ 20	38 (38/152, 25.0%)
21 ~ 30	23 (23/152, 15.1%)
31 ~ 40	10 (10/152, 6.6%)
41 ~ 50	17 (17/152, 11.2%)
51 ~ 60	16 (16/152, 10.5%)
61 ~	10 (10/152, 6.6%)
Male/Female, *n* (Male %)	93/59 (61.2%)
Clinical variables	
Fever duration (day), mean ± SD	6.1 ± 2.9
Eschar, *n* (%)	144 (144/152, 94.7%)
Rash, *n* (%)	3 (3/152, 2.0%)
Myalgia, *n* (%)	25 (25/152, 16.4%)

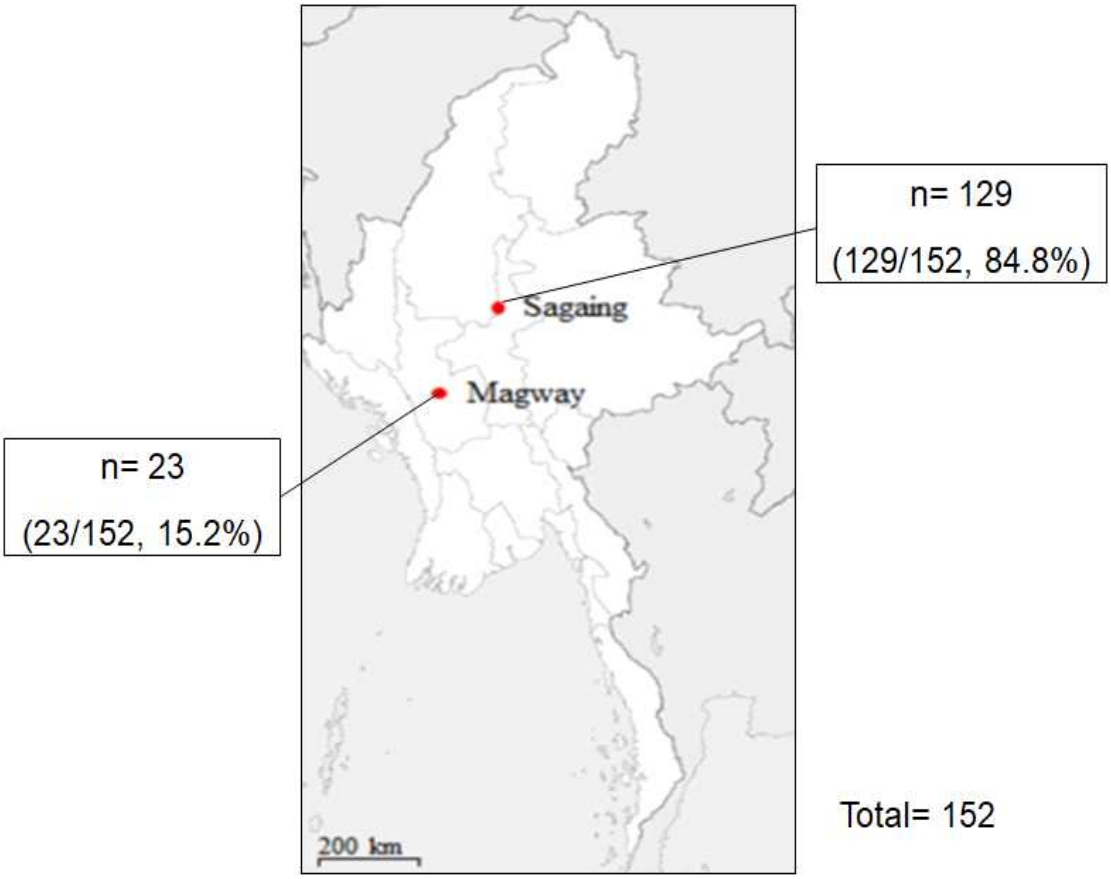

Figure 9. Map of Myanmar showing the locations of Sagaing and Magway provinces where patients' sera were collected

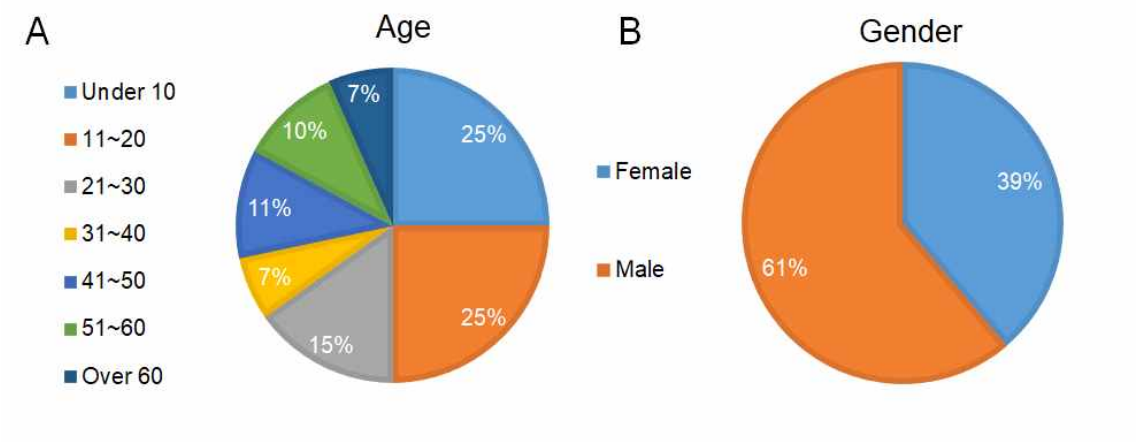

Figure 10. Characteristics of 152 patients' sera with potential scrub typhus in Myanmar. (A) Patient age distribution, (B) Patient gender distribution

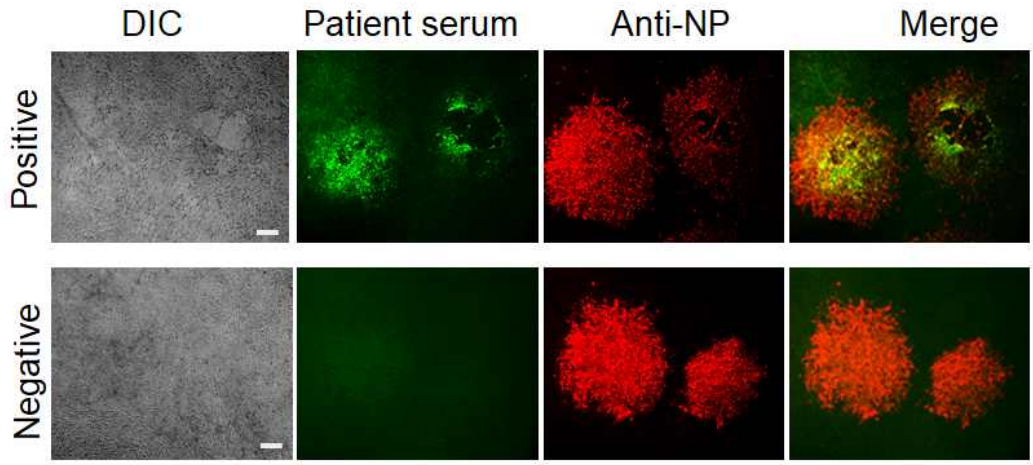

Figure 11. Immunofluorescence assay (IFA) to detect SFTSV-specific IgG antibody responses in patients' sera in Myanmar. SFTSV plaque was stained with the patients' sera with a serial dilution of the patients' sera titer and rabbit anti-SFTSV NP antibody as an internal positive control. The upper image is a positive patients' serum that presented the presence of SFTSV-specific IgG antibody response. The lower one is a negative sample that showed the absence of the presence of SFTSV-specific IgG antibody response. SFTSV plaque is stained by patient serum shown by green color, SFTSV plaque is stained by a rabbit anti-SFTSV NP antibody shown in red color. DIC: Differential interference contrast. Bar: 100μm.

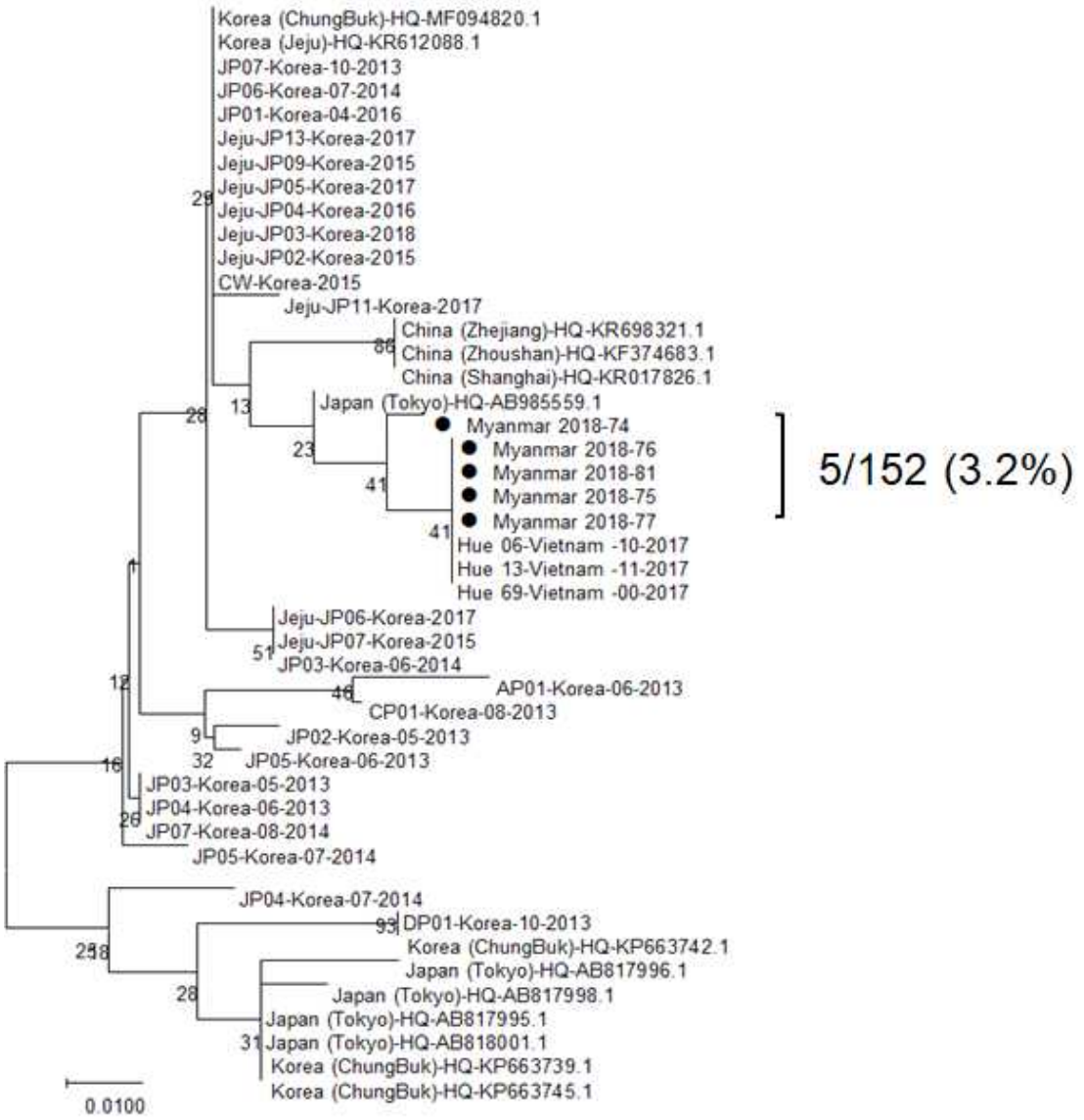

Figure 12. Phylogenetic tree of partial S sequences of SFTSV in Myanmar. The tree was constructed using the maximum likelihood method with MEGA6. The partial the S sequences amplified from the sera of indicated patients were analyzed and shown in black circle and compared with 40 representative genotype sequences. The partial S sequence data for the viruses identified in China, South Korea, and Japan were obtained from NCBI/BLAST.

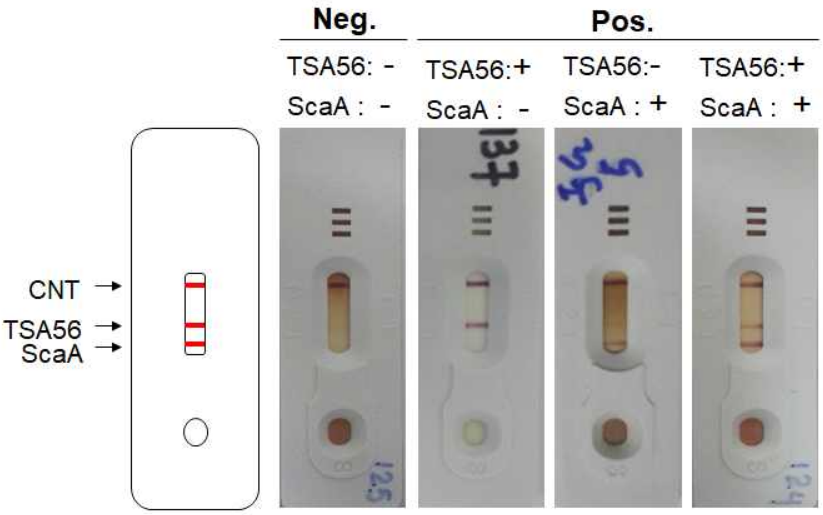

Figure 13. Initial serological diagnosis of patients' sera in Myanmar by Immunochromatography test (ICT) strip. Representative images of ICT results detecting specific IgG against TSA56 and ScaA antigen in suspected scrub typhus patients' sera. The red band appearing on the control line (C) and test line (T) including TSA56 and ScaA line concurrently or either way was regarded as a positive (Strip 3,4,5). The test was considered as a negative when only the control band appeared as red (Strip 2). And if no band appeared on the control line, then the test was considered invalid.

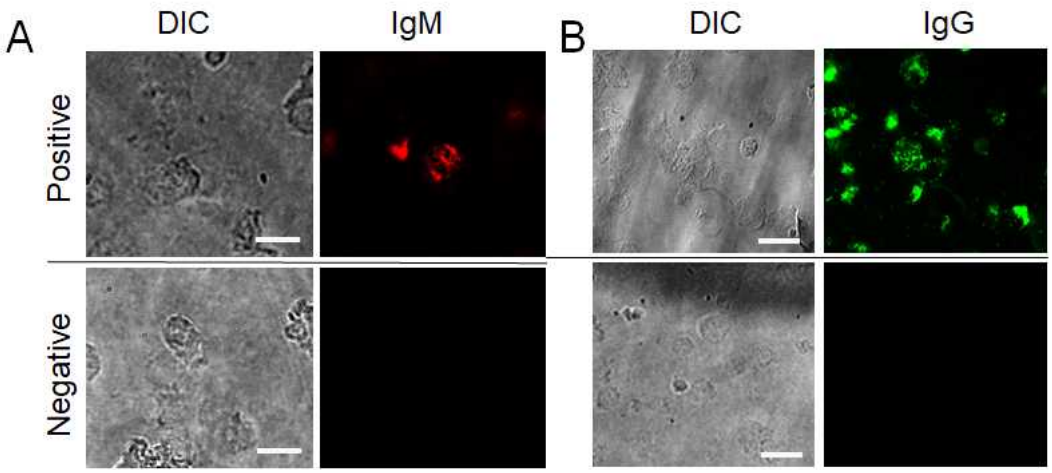

Figure 14. Antibody response specific to *O. tsutsugamushi* in patients' sera in Myanmar. (A) *O. tsutsugamushi*–specific IgM antibody test. (B) *O. tsutsugamushi*–specific IgG antibody test. The upper image is a positive patients' serum that presented antibody specific for *O. tsutsugamushi*. The lower one is a negative sample that showed the absence of antibody–specific for *O. tsutsugamushi.* DIC: Differential interference contrast. *O. tsutsugamushi* (IgM–red color, IgG–green color). Bar: 20μm.

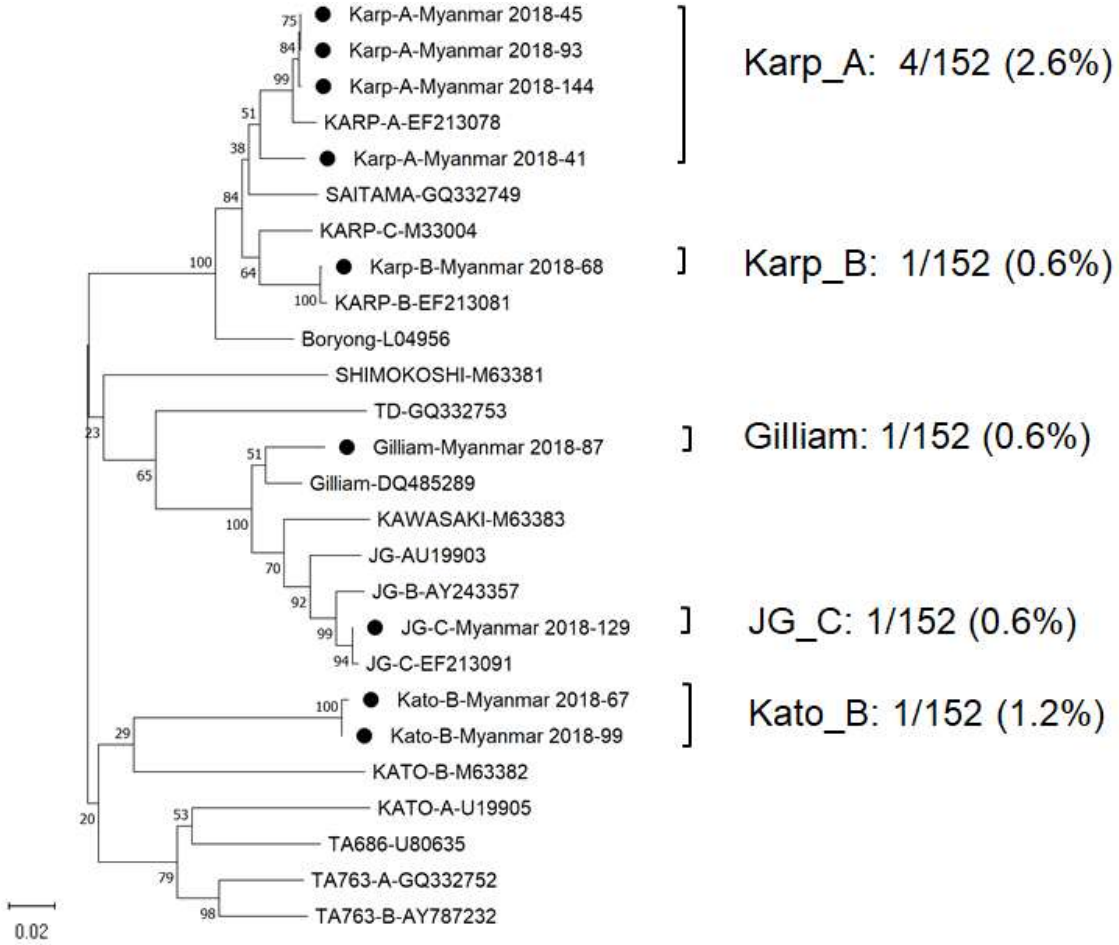

Figure 15. Phylogenetic tree of *O. tsutsugamushi*-specific *tsa56* gene sequences in Myanmar. The tree was constructed using the maximum likelihood method with MEGA6. The *tsa56* gene sequences identified in this study are indicated by full black circles and compared with 17 representative genotype sequences. The *O. tsutsugamushi tsa56* gene sequence data identified from China, South Korea, Japan, and India were obtained from NCBI/BLAST.

Table 5. Summary of diagnosis results using patients' sera in Myanmar

Pathogen	Method	2018	# of positive samples
SFTSV	IFA		1/152 (0.65%)
	PCR		5/152 (3.3%)
O. tsutsugamushi	ICT	anti-TSA56 IgG positive (%)	36/128 (28.1%)
		anti-ScaA IgG positive (%)	25/128 (19.5%)
		anti-TSA56 + anti-ScaA IgG positive (%)	20/128 (15.6%)
	IFA	IgG positive (%)	119/152 (78.3%)
		IgM positive (%)	90/152 (59.2%)
		IgG + IgM positive (%)	69/152 (45.3%)
	PCR		9/152 (5.9%)

Table 6. Potential coinfection with *O. tsutsugamushi* and SFTSV in Myanmar patients

Diagnosis criteria	# of positive samples
SFTS (RT-PCR +) / scrub typhus (IFA +)	5/152 (3.3%)
SFTS (IFA +) / scrub typhus (IFA +)	1/152 (0.7%)

DISCUSSION

Severe fever with thrombocytopenia syndrome is a newly emerging viral disease in Asia that infects humans primarily through tick bites. Transmission from person to person has been confirmed in people with close contact such as family members and health-care workers, and asymptomatic infection via close contact from person to person has also been reported.

Scrub typhus is an acute febrile illness caused by *O. tsutsugamushi*, a bacterium transmitted to humans through chigger mite (Acari: Trombiculiidae) bites. The vectors of SFTSV and *O. tsutsugamushi* are different; *H. longicornis* is a major vector of SFTSV and *Leptotrombidium* spp. mites are vectors of *O. tsutsugamushi*. However, a previous report showed that SFTSV was detected by RT-PCR in *L. scutellare* mite bites in China, suggesting that the mites are a potential vector for SFTSV; Wi et al. suggested the possibility of the coinfection with SFTSV and *O. tsutsugamushi* in South Korea. However, there has been no report of mixed infection with both SFTSV and different genotypes of *O. tsutsugamushi* in patients. In this study, I retrospectively confirmed 15 SFTSV infections or mixed infection with *O. tsutsugamushi* by amplification of the partial S segment of the viral RNA and the bacterial DNA, as well as detection of IgM and/or IgG antibodies against SFTSV and *O. tsutsugamushi* from the stored sera of patients who had an acute febrile illness from November 1, 2000, to November 1, 2003, in South Korea. Although the case-fatality rate of scrub typhus is low due to antibiotic treatment, severe scrub typhus remains an unresolved issue. Due to the potential for a severe clinical form, a clinical prediction tool will be useful to narrow the differential diagnosis for those requiring further urgent investigation. 15 samples (15/37, 40.5%) were positive via IFA (≥1:160). 9 samples (9/37, 24.3%) detected the specific PCR products *NP* gene. The number of infected patients with positive IFA had rapidly increased each year from 2000-2003. The total number of patients in the year 2000 was 6. The year 2001 had 0 patients among the tested samples. The year 2002 had 3 infected patients. But the number of patients in the year 2003 dramatically increased to 6 patients out of 2,328 samples. Severe fever

with thrombocytopenia syndrome virus was previously reported in South Korea in 2010. Therefore, I suggest that SFTSV infections in South Korea occurred before the previously reported cases and were more concurrent with those in China.

Coinfection of *O. tsutsugamushi* with several other pathogens has been reported. Diseases shown to occur concurrently with scrub typhus include pneumonia caused by Mycoplasma pneumoniae[52], murine typhus[53], Q fever[54], leptospirosis[55], chicken pox[56], malaria[57], and dengue[58]. Several of the coinfection pathogens are susceptible to the antimicrobials used to treat scrub typhus. However, a coinfection should be suspected in scrub typhus patients if defervescence does not occur within 48–72 hours of appropriate antimicrobial treatment and combination therapy may be required[59][60]. Unusually, one *O. tsutsugamushi* coinfection may be advantageous to the patient. The clinical evidence on the possibility of the coinfection of *O. tsutsugamushi* and SFTSV is not solid, and further monitoring and study are necessary. From an ecological perspective, given the vectors of the two diseases, coinfection is rare. Although the epidemic seasons overlap and there is a risk of simultaneously acquiring the two diseases during outdoor activity, they do not share vectors, and the ecologies of their vectors differ. Phenotypically, scrub typhus is highly prevalent in the rice field areas of western and southwestern South Korea, whereas the incidence of SFTS is low in this area, consistent with the low SFTSV is high in the eastern and southeastern mountainous area of SFTS is low in this area, consistent with the low of SFTSV infection rate in ticks. Conversely, the incidence of SFTSV is high in the eastern and southeastern mountainous areas of South Korea[61]. In South Korea, following hospital records, by IFA method, some patient samples were positive with Hantavirus which caused Hemorrhagic fever and renal syndrome (HFRS), *Leptospira* caused Leptospirosis, and *R. typhi* caused murine typhus. Among 37 tested samples, there were 1 samples which were positive with *Leptospira* and 4 samples positive with *R. typhi* (Table 1). 2 samples (2/37, 5.4%) were positive with SFTSV and *R. typhi* (≥1:1,280) in 2001. There was 1 sample (1/37, 2.7%) positive for all SFTSV and *O. tsutsugamushi* (≥1:2,560) and *Leptospira* (≥1:160) and 1 sample (1/37, 2.7%) was positive for all SFTSV and *O. tsutsugamushi*

(≥1:2,560) and *R. typhi* (≥1:160) in 2002. There was 1 sample (1/37, 2.7%) positive for SFTSV and *R. typhi* (≥1:80) in 2002. The possibility of coinfection with *O. tsutsugamushi* and another pathogen should also be considered, SFTSV is also can be coinfected with *O. tsutsugamushi*.

Based on the results of the genetic detection of the two pathogens, among them 9 samples detected PCR product, there were 4 samples (4/37, 10.8%) which showed coinfection between *O. tsutsugamushi* and severe fever with thrombocytopenia syndrome virus (SFTSV) by detecting both the SFTSV *NP* gene and *O. tsutsugamushi*-specific *tsa56* gene. 4 samples showed coinfection between 2 kinds of the pathogen (Karp and Gilliam) which were all classified under the genotype of *O. tsutsugamushi*. 15 patients' sera (15/37, 40.5%) showed a positive result by staining the SFTSV plaque (≥1:160). 5 of 15 samples (5/37, 13.5%) were positive for both pathogens *O. tsutsugamushi* and SFTSV via the IFA method with titer ≥1:1,280 and ≥1:160, respectively. The coinfection could happen in both pathogens *O. tsutsugamushi* and SFTSV. The coinfection could happen in both pathogen *O. tsutsugamushi* and SFTSV. The percentage of potential coinfection with *O. tsutsugamushi* and SFTSV is high and I can detect the pathogen-specific antibody responses at a high titer of antibody dilution. Sometimes patients had a primary infection, the initial infection caused by one pathogen such as SFTSV or *O. tsutsugamushi*, which can continue with secondary infection by a different pathogen (SFTSV or *O. tsutsugamushi*). It was considered as evidence for potential coinfection with the two pathogens *O. tsutsugamushi* and SFTSV. This study showed that the population over 60 years old included the highest number of patients at 14 (14/37, 37.8%) compared to other age groups. The 37 patients investigated included 10 males (37.03%) and 27 females (72.97%). Due to a lack of information about the living area of the patients, I can give a hypothesis that was similar to the findings in other Asian areas[62)63)]. Most infected farmers were elderly women ≥60 years old. These findings might be explained by the unbalanced development between rural and urban areas, which results in the problem of left-behind children, the elderly, and women. It included a higher probability of exposure to mites, ticks, or domestic animals with SFTSV and *O. tsutsugamushi*, and the frequency of latent infections or asymptomatic patients with both pathogens varied.

Person-to-person transmission of SFTSV has been demonstrated in both China and Korea. In these instances, the disease is typically fatal, with a high viral load in the blood observed following infection by contact with contaminants[64)65)].

In South Korea, scrub typhus is a major public health problem during the harvest season in South Korea (October and November); 10,485 cases were reported in 2013[57)]. In my study, the season with the highest number of patients positive for the SFTSV NP-specific antibody was November with 25 samples (25/37, 67.5%). In October and December, there were 5 positive samples (5/37, 13.5%) and 3 positive samples (3/37, 8.1%), respectively. The incidence of SFTS showed obvious seasonal characteristics, having an overlap of seasonality between scrub typhus and SFTS.

The time point to collect the patients' sera can determine the possibility of detection of the pathogen-specific IgG and IgM antibodies. In the South Korea surveillance, no samples could be detected with SFTSV NP-specific IgM antibodies. SFTSV NP-specific IgG antibodies were detected in the sera of 37 patients (37/2,328, 1.546%). 37 sera samples positive for anti-SFTSV NP IgG by ELISA were tested for IgG antibodies against *O. tsutsugamushi* using IFA. The IFA results used to detect IgG antibodies showed that *O. tsutsugamushi* specific IgG antibodies were detected in the sera of the 17 patients (17/37, 45.9%). No sample can be detected for the *O. tsutsugamushi* specific IgM antibody via IFA method.

9 samples showed a clear genotype for *O. tsutsugamushi* with the 3 genotypes Karp_C (6/37, 16.2%), Boryong (1/37, 2.7%), Gilliam (2/37, 5.4%). 5 samples showed a mixed-infected *O. tsutsugamushi* strain (5/37, 13.5%). By TA cloning, I was only capable of classifying strains isolated from mixed-infected *O. tsutsugamushi* in 2 samples. Both of them were mixed between Karp and Gilliam strains. In South Korea, the Boryong genotype is more prevalent. But in this study, Karp_C was shown to be more abundant than the other genotypes such as Boryong. This study could not represent the genotype distribution of *O. tsutsugamushi* in Korea.

The signs and symptoms of SFTS and scrub typhus are similar, but SFTS exhibits a higher mortality rate than scrub typhus. Therefore, it is important to consider the possibility of SFTSV infection in patients having

scrub typhus potentially being coinfected with SFTSV. Thus, there is a need to better understand the ecological transmission dynamics and geographic distribution of SFTSV and *O. tsutsugamushi* in endemic countries.

Moreover, this study investigated the serological prevalence and genotypic diversity of *O. tsutsugamushi* in clinically-suspected scrub typhus patients in Myanmar for the first time since the 1940s. Sagaing province and Magway province are regions of Myanmar. In these two provinces, agriculture is the major occupation, including the growing of rice, wheat, sesame, peanut, tobacco, sesamum, and groundnuts. Patients' work-related information was not surveyed in this study but scrub typhus in the southern parts of Korea and Japan was more prevalent in farmers or people involved in agricultural work[66]. Among the 152 suspected patients that suffered from the acute febrile illness, eschar was observed in 144 patients (144/152, 94.7%) and serological positive responses, either specific IgG and IgM against the bacterial pathogen, was 78.3% and 59.2%, respectively, as assessed by via IFA. Since I was only able to examine sera collected during the acute phase of infection and could not assess the rise of antibody titers in paired samples in the convalescent phase, I was not able to confirm the exact prevalence of scrub typhus in the suspected patients. Nevertheless, the relatively high prevalence of eschar, implying mite-biting, and high titers of antibodies against *O. tsutsugamushi* suggests that scrub typhus is still prevalent in Myanmar as previously reported in the 1940s[67][68]. Whilst eschar, which is the vasculitic skin reaction following bites from the infected chiggers, may assist in the diagnosis of scrub typhus, not all cases were accompanied by skin lesions in the setting of multiple exposures with chigger bites, the incidence of eschar is much less due to the impaired cellular immunity of the host[74]. Several recent studies conducted on the Thai-Myanmar border also identified scrub typhus as one of the major Rickettsioses among a mixed population of migrant workers, including those from Myanmar[69]. Of note, a high prevalence of scrub typhus in children was confirmed in two separate studies, as I also observed during this work. Therefore, young children with febrile illness should be carefully observed for early diagnosis and treatment of scrub typhus in the endemic area of Southern Asia[70], and the

baseline levels of antibody titers against *O. tsutsugamushi* in healthy individuals need to be assessed to determine the cut-off titers for acute scrub typhus in the endemic region[71].

Early clinical diagnosis of scrub typhus is hampered by difficulty in differentiating from other febrile infectious diseases. Moreover, performing sophisticated laboratory tests, such as IFA using bacterial cultures, is not feasible for scrub typhus in resource-limited settings, especially in many of the endemic countries of Southern Asia; accurate point-of-care testing (POCT) would be invaluable for patient diagnosis and management[72]. Here, I applied ICT to detect the prevalence of antibody responses against TSA56 and ScaA in patients as a rapid diagnostic method for point-of-care testing (POCT). 20 patients' sera (20/36, 55.6%) showed positive IgG responses against both antigens and 5 samples (5/36, 13.9%) were positive for the ScaA antigen only, suggesting potential applicability of the ScaA antigen for the diagnosis of scrub typhus when simultaneously used with the TSA56 antigen, a dominant immunogen of *O. tsutsugamushi* [73]. However, the overall positive rate of the home-made ICT for acute infection was relatively low (41/128, 32%) when compared to IFA detecting IgG responses against the bacterial antigen (101/128, 78.9%). In a recent assessment of the diagnostic accuracy of commercially available ICT kits, the sensitivity and specificity were 20.9-23.3% and 74.4-81.4%, respectively, for acute specimens and 32.6-76.7% and 76.7-79.1%, respectively, for convalescent specimens[74]. The poor sensitivity of commercial ICTs and my current study when performed on acute specimens highlights the difficulties in prompt diagnosis of scrub typhus. Future research should be directed towards creating a new ICT method with an optimal combination of antigens from locally prevalent genotypes and/or nucleic acid-based detection[75] to improve diagnostic accuracy in the early period of disease progression. Scrub typhus should remain high on the list of differential diagnosis when faced with undifferentiated clinical symptoms and signs as clinical parameters alone hardly discriminate between scrub typhus and others, thus a need for a high index of clinical suspicion coupled with reliable confirmatory tests. Rapid tests are promising which may aid in early diagnosis; however more studies are required to evaluate its reliability in endemic regions.

Genotyping *O. tsutsugamushi* by using amplified sequences of *tsa56* genes revealed that at least five different genotypes (Karp_A (44.4%), Karp_B (11.1%), Kato_B (22.2%), Gilliam (11.1%), and JG_C (11.1%), are currently present in central Myanmar. In an earlier study using sera from 15 scrub typhus patients in Myanmar (Burma) and the Philippine islands in 1944 [76], complement fixation tests typed the isolates as Gilliam (36%), Karp (7%), Seerangayee (7%), and mixed strains (50%). Another recent study also reported the presence of Gilliam and Karp strains in 3 patients of the central Thai–Myanmar border, as assessed by serotyping. My current study revealed genetic heterogeneity in Myanmar patients, similar to those observed in nearby countries including Bangladesh[77], Northern Thailand[78], and Laos[79]. This study highlights the importance of considering scrub typhus as a diagnostic possibility in cases of acute undifferentiated febrile illness in rural areas. Since then, there have been no reports describing the prevalence and genetics of *O. tsutsugamushi* in Myanmar. Scrub typhus is likely to remain an under–appreciated and under–diagnosed disease, which may be due to non–specific clinical presentation and the laborious nature of its diagnosis requirement.

SFTS is a tick–borne viral disease endemic in Eastern Asia and has been reported in China, South Korea, Japan, and Vietnam. Recently, serological and genetic surveillance of SFTSV using sera from domestic animals and ticks *(R. microplus)* also showed SFTSV RNA and antibodies in animals and ticks in Taiwan[80]. Of note, the Taiwan Centers for Disease Control (Taiwan CDC) announced the first human case of SFTS on November 19, 2019 (https://www.cdc.gov.tw/Bulletin). SFTSV has been found in humans, ticks, and animals. The anti–SFTSV antibodies positive ratios in domestic and wild animals were 43.58% (163/374) and 46.47% (112/241), respectively. SFTSV has a wide host range and infections in some animal species. The pooled seroprevalence of anti–SFTSV antibodies was 45.70% in goats and sheep, 36.70% in cattle, 29.50% in dogs, 9.6% in chickens, 3.2% in rodents, and 3.2% in pigs. SFTSV RNA was also detected in blood samples from several animal species, with a carriage rate varying from 0.23 to 26.31%. The highest carriage rate of SFTSV RNA was detected in cattle (up to 26.31%), followed by cats (17.46%), goats (9.1%), and rodents (8.44%), but there is no evidence that the virus can cause disease

in animals. The intimate contact with animals increases the probability of a tick bite and this then increases the chances of SFTSV infection, and besides, contact with the secretions of infected animals may also be a route of transmission leading to SFTV infection[90]. Zhang and Xu (2016) reported that the status of SFTSV infection from domestic and wild animals was related to transmission from different tick species. They proposed a tick and migratory bird model for SFTSV transmission and a hypothesis for the local transmission and wide dissemination of SFTSV by combining infection data with host and vector ecology data. They also showed that the risk of infection is reduced if the animals and vector populations are monitored[81].

Here, I retrospectively confirmed five SFTSV infections and mixed infection with *O. tsutsugamushi* by amplifying the partial S segment of the viral RNA and detection of IgM and/or IgG antibodies against *O. tsutsugamushi* from the sera of patients with acute febrile illness in Myanmar for the first time. The SFTSV-positive patients did not travel abroad and all five positive patients live in the same village in Sagaing province, suggesting that there are hot spots for SFTSV infection. Several different tick species, such as *H. longicornis*, *R. microplus, A. testudinarium* and *I. nipponensis* are reported as potential vectors for the transmission of SFTSV, although *H. longicornis* is known as the primary vector[82].

The presence of *R. microplus* and *A. testudinarium* has been reported in Myanmar [83]. Migratory birds might also function as long-distance carriers of virus-bearing ticks and disseminate SFTSV from endemic countries to tropical Southern Asia. Another important issue is the coinfection of SFTSV and *O. tsutsugamushi*. The primary vectors for scrub typhus are *Leptotrombidium* spp., especially *L. deliense* in south Asian Asian countries including Myanmar[84]. The coinfection of *O. tsutsugamushi* and SFTSV might be mediated by either simultaneous infestation by two different vectors carrying each pathogen or by a single tick or mite species carrying both pathogens. In China, SFTSV has been detected in *L. scutellare* mites[85] and the coinfection of SFTSV with various bacterial pathogens has been reported in hard ticks, including *H. longicornis*[86]. A previous report showed that SFTSV was detected by RT-PCR in *L.*

scutellare mite bites in China, suggesting that the mites are a potential vector for SFTSV which suggests the possibility for coinfection with SFTSV and *O. tsutsugamushi*. In another study in China, the presence of tick-borne pathogens and their possible coinfections were evaluated among host-seeking ticks in seven cities from Jiaodong peninsula, Shandong Province, with specific PCR or reverse transcription-PCR tests. Coinfection of *A. capra* and SFTSV was also discovered from two female *H. longicornis* in Pingdu city. These results indicated that the potential human pathogens other than severe febrile and thrombocytopenic syndrome might be transmitted by hard ticks separately or in combination[91].

Besides, coinfections of different tick-borne bacterial and viral pathogens in both humans and animals have been commonly reported in recent years [87][88][89]. Notably, coinfection with spotted fever group rickettsiae was identified in 77 of 823 patients infected with SFTSV in a study from China, and the coinfection resulted in delayed recovery and increased risk for death. In this study, all five SFTSV-positive patients recovered within a week, and four of them were less than 15 years old. Given that the disease severity of SFTS is significantly associated with the host' s age and the viral genotype[90], milder clinical symptoms observed in the Myanmar patients might be due to earlier exposure and/or prevalence of less virulent genotypes of SFTSV in Myanmar. Therefore, continuous surveillance of SFTSV patients with detailed clinical manifestations and associated viral genotypes prevalent in the local area needs to be conducted. Coinfection contributes significantly to the diagnostic challenges of acute febrile illnesses, especially in endemic areas. The confusion caused by overlapping clinical features impedes timely management. In addition, more reliable differential diagnosis techniques and rational prevention and control measures are required for better clinical practices in the endemic regions of multiple ticks and mite-borne pathogens[91][92]. Further clinical, epidemiological, and laboratory research is also needed to better understand the ecological transmission dynamics and geographic distribution of SFTSV and *O. tsutsugamushi*[93][94].

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국문 초록

쯔쯔가무시병은 오리엔시아 쯔쯔가무시 (*Orientia tsutsugamushi*)에 의한 감염으로 발생하는 급성열성 감염질환이다. 중증 열성 혈소판 감소 증후군 바이러스 (Severe Fever with Thrombocytopenia Syndrome Virus) 는 진드기 매개 바이러스로, 출혈열을 유발한다. 중증 열성 혈소판 감소 증후군은 중국, 남한, 일본 등 동아시아에서 나타나고 있으며, 이는 쯔쯔가무시병이 발생하는 지역과 일치한다. 쯔쯔가무시병과 중증 열성 혈소판 감소증이 역학적, 임상학적으로 중첩되기 때문에 이 매개체 감염 질환들의 지역적인 발병에 대한 이해는 목표지향적 조사와 치료 계획 수립에 있어 매우 중요하다.

방법: 본 연구에서는 오리엔시아 쯔쯔가무시의 유전적 다양성, 그리고 한국과 미얀마 내 오리엔시아 쯔쯔가무시와 중증 열성 혈소판 감소 증후군 바이러스와의 잠재적 동시감염을 조사하고자 하였다. 이를 위하여 2000년부터 2003년까지 발병한 한국의 열성 질환 환자 혈청 2,328개, 리케치아성 질환으로 진단받은 미얀마 환자 152명의 혈청을 조사하였다. 쯔쯔가무시병과 중증 열성 혈소판 감소 증후군에 대한 혈청학적, 분자적 검사를 시행하였으며, 두 병원체의 유전형은 혈청 시료에서 증폭된 각 병원체의 유전자 시퀀싱을 통해 확정되었다.

결과: 한국의 2328개의 시료 중 14개에서 오리엔시아 쯔쯔가무시의 tsa56 특이적인 유전자 증폭 산물을 발견하였으며, 보령 (1/37, 2.7%), 카프 (8/37, 21.6%) , 길리암 (4/37, 10.8%) 등 최소 4가지 유전형이 존재함을 밝혀내었다. 나아가 9개의 시료에서는 SFTSV의 NP 유전자 특이적인 유전자 증폭 산물이 탐지되었다. 9개 중 3개의 시료에서 두 병원체 유전자가 동시에 확인 되었다. 미얀마 환자의 혈청에서는 152개의 시료 중 9개의 시료에서 카프 A (4/9, 44.4%), 카프 B (1/9, 11.1%), 가토 B (2/9, 22.2%), 길리암 (1/9, 11.1%), 그리고 JG_C (1/9, 11.1%) 등 5개의 쯔쯔가무시균 유전형이 발견되었다. 또한 5개의 시료에서 SFTSV의 np 유전자 특이적인 유전자 증폭 산물이 탐지되었다. 5개의 SFTSV 유전자 양성시료는 모두 쯔쯔가무시균 유전자 음성이었으나, 세 개의 혈청시료에서 쯔쯔가무시균에 대한 항체가 높은 역가(>1:2560)로 관찰되었다.

결론: 2000년부터 2003년까지 한국 열성질환 환자, 그리고 2018년 수집된 미얀마의 리케치아 질환 의심 환자의 혈청 시료에서 혈청학적, 분자적 추적조사를 통하여 쯔쯔가무시병과 중증열성혈소판감소증의 혈청학적 유병률과 유전자 양성률을 확인하였다. 또한 한국과 미얀마에서 유행하는 쯔쯔가무시균의 유전형 다양성을 확인하였으며, 쯔쯔가무시균과 중증열성혈소판감소증후군 바이러스의 동시감염을 진단하

였다. 따라서 초기 증상이 유사한 절지동물매개 감염질환의 감별진단과 유행하는 유전형 다양성에 대한 보다 자세한 조사를 통해 유행지역에서의 진단과 치료 전략을 수립하는데 필요한 정보들을 확대할 필요성이 있음을 제안하고자 한다.

주요어: **쯔쯔가무시병, 혈소판 감소증 바이러스,동시 감염**
학번: 2014-25232